

**New insights into androgen-dependent Wolffian duct
development**

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Declaration

The studies in this thesis were the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification.

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Abstract

In mammals, male and female fetal reproductive tracts are initially indistinguishable with Wolffian ducts (WD) and Müllerian ducts (MD) present in both sexes. Androgens play a vital role in masculinisation of the fetus, including WD rescue and development in males; however, the mechanisms that underlie this process are unknown. The aims of the current study therefore were to investigate the timing and mechanisms involved in androgen mediated WD development. The present study exposed pregnant rats to the androgen receptor antagonist, flutamide (50 or 100 mgkg⁻¹), Di(n-butyl) phthalate (DBP; 500 mgkg⁻¹) which reduces testicular testosterone production, and/or exogenous testosterone during specific time windows in fetal life in order to establish the key time windows for androgen-regulated WD development and the possible mechanisms involved.

These studies confirmed the vital role for androgens in WD development and highlighted their critical involvement in establishing the early patterning of WD development between e15.5-17.5, prior to any sign of morphological differentiation. At this stage, androgens receptors (AR) are only expressed in the stroma, not in the epithelium, thus androgens must regulate WD differentiation via stroma-epithelial interactions. Contrastingly, high levels of androgens were not required during morphological differentiation of the WD between e19.5-21.5 (when coiling occurs). Impaired androgen action during the correct window of development disrupted WD development as evidenced by reduced coiling of the future epididymis. This was likely due to a demonstrated reduction in cell proliferation in both stromal and epithelial compartments, impaired stromal differentiation, reduced epithelial cell height and ultimately epithelial degradation. These stromal abnormalities were noted prior to observing any obvious abnormalities in the epithelium, further highlighting regulation of the epithelium by the stroma. The mechanisms involved in this impaired WD development include interruption to the basement membrane and extracellular matrix, as evidenced by altered expression of some intermediate filaments. These were similar to the mechanisms noted in the regressing female WD but impaired androgen action did not induce apoptosis in the male WD, which was observed in the regressing WD in females. Maternal exposure to testosterone during gestation was able to rescue the female WD and even induce some degree of morphological differentiation, although this was to a lesser degree than that noted in the normal male.

In adult males similarly exposed to flutamide in utero, the majority (80%) of WD-derived tissues were absent, compared to no incomplete WDs at e19.5 and 11% at e21.5. This demonstrates that the major impact of flutamide is on epididymal differentiation rather than its initial stabilisation, as had previously been assumed. This increased prevalence of epididymal abnormalities was noted after puberty and is likely to be a result of failure to establish normal patterning of the WD in the fetus.

Further reducing androgen action either in the Tfm mouse, where the AR is inactivated, or by exposing rats to DBP plus flutamide *in utero*, resulted in a complete loss of WD tissue by birth. However, further investigation highlighted that the male WD regressed later in these models than occurs naturally in females thus suggesting a possible role for a compensatory mechanism in the absence of androgen action. It is important to note though that this mechanism is not sufficient alone to maintain the WD long term or to support full differentiation in the male.

In conclusion, androgens alone appear to be sufficient for WD development but in their absence, a compensatory mechanism may try to rescue the male WD. Fetal WD differentiation is far more susceptible to blockade of fetal androgen action than is its initial stabilisation although these effects appear to be mediated by disruption of stromal-epithelial interactions during the stabilization period. Persistence of WD-derived structures postnatally is also affected, however, this is likely to be due to impaired patterning of the fetal Wolffian duct rather than prevention of initial stabilisation. These findings have identified the critical window for androgen action in the WD and thus have created an opportunity to study the elusive mechanisms behind androgen action. Since androgens act on many other systems in the body with many parallels existing between the male and female reproductive system, the outcomes of these studies may therefore impact on our understanding of WD development but also of other androgen-dependent processes such as the development, function and diseases of other reproductive tissues.

Presentations relating to this thesis

The androgen-dependent mechanisms of Wolffian duct differentiation and their perturbation by flutamide and DBP.

Poster presented at the 3rd Copenhagen workshop on Environment, Reproductive health and Fertility, Copenhagen, Denmark, January 2005

Mechanisms of Wolffian duct differentiation; evidence for androgen-driven stromal epithelial interactions.

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Mechanisms of interrupted Wolffian duct differentiation after exposure to environmental anti-androgens; comparative studies using flutamide and n-dibutyl phthalate (DBP).

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Investigation of Wolffian duct stabilisation and its dependence on androgens; a comparison of WD regression in females and the effects of androgen-withdrawal in males. (*in submission*)

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Abbreviation	Definition
17 β HSD	17 β -hydroxysteroid dehydrogenase
ABC	avidin/biotin complex
AGD	anogenital distance
AIS	androgen insensitivity syndrome
AMH	Anti-Mullerian hormone
AR	androgen receptor
ARE	androgen response element
ARKO	androgen knockout
ArKO	aromatase knockout
CAH	congenital adrenal hyperplasia
CAIS	complete androgen insensitivity syndrome
DAB	3,3 DiAminoBenzidine
DBD	DNA-binding domain
DBP	di-n-butylphthalate
DES	diethylstilboestrol
Dhh	desert hedgehog
DHT	dihydrotestosterone
e	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transformation
ERKO	oestrogen receptor knockout
ER α	oestrogen receptor α
ER β	oestrogen receptor β
FGF	fibroblast growth factor
Fst	follicle-stimulating hormone
Gata4	GATA binding protein 4
GR	glucocorticoid receptor
h	hour
H&E	haematoxylin and eosin
Hox	homeobox transcription factors
HSP	heat shock protein
IF	intermediate filament

IGF-1	insulin-like growth factor
IGF-1R	insulin-like growth factor 1 receptor
Insl 3	insulin-like protein 3
IUGR	intrauterine growth restriction
LBD	ligand-binding domain
KGF	keratinocyte growth factor
MD	Müllerian duct
min	minute
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NGS	normal goat serum
PAIS	partial androgen insensitivity syndrome
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PDGF	platelet derived growth factor
PFA	Paraformaldehyde
PGD2	prostaglandin D2
Pnd	postnatal day
RXR	retinoid X receptor
s	second
S.E.M.	standard error of the mean
SF-1	nuclear receptor subfamily 5
SMA	smooth muscle actin
Sox 9	Sry-like HMG-box protein 9
Sry	sex determining region of the Y chromosome
T	testosterone
TBS	tris buffered saline
Tfm	testicular feminised mice
TP	testosterone propionate
UGS	urogenital sinus
UGT	urogenital tract
WD	Wolffian duct
Wnt 4	wingless-related MMTV integration site 4
Wnt7	wingless-related MMTV integration site 7
WT-1	Wilms tumour homologue

1 Literature review

1.1 Sex determination

Sexual differentiation is a complex, pre-programmed series of events regulated by both genetic and hormonal mechanisms to transform and differentiate the indifferent gonads and the internal and external sex structures into male and female specific structures. In mammals, the reproductive system consists of the primordial gonad, the two genital duct systems (namely the Müllerian and Wolffian ducts), and a common opening for genital ducts and the urinary tract to the outside through the genital folds. Initially, this tract is morphologically indistinguishable in male and female embryos but is determined by genetic sex, which is dictated at conception by the inheritance of an X or Y chromosome from the male gamete. These sex chromosomes promote the differentiation of the indifferent gonad into a testis or ovary, which subsequently drives differentiation of the internal and external sex structures via hormone production.

It is important to note that during sexual differentiation, each component of the urogenital system undergoes various developmental changes to generate a male or a female. The precise window for this differentiation is distinct for each structure and is highlighted in Fig. 1.1.

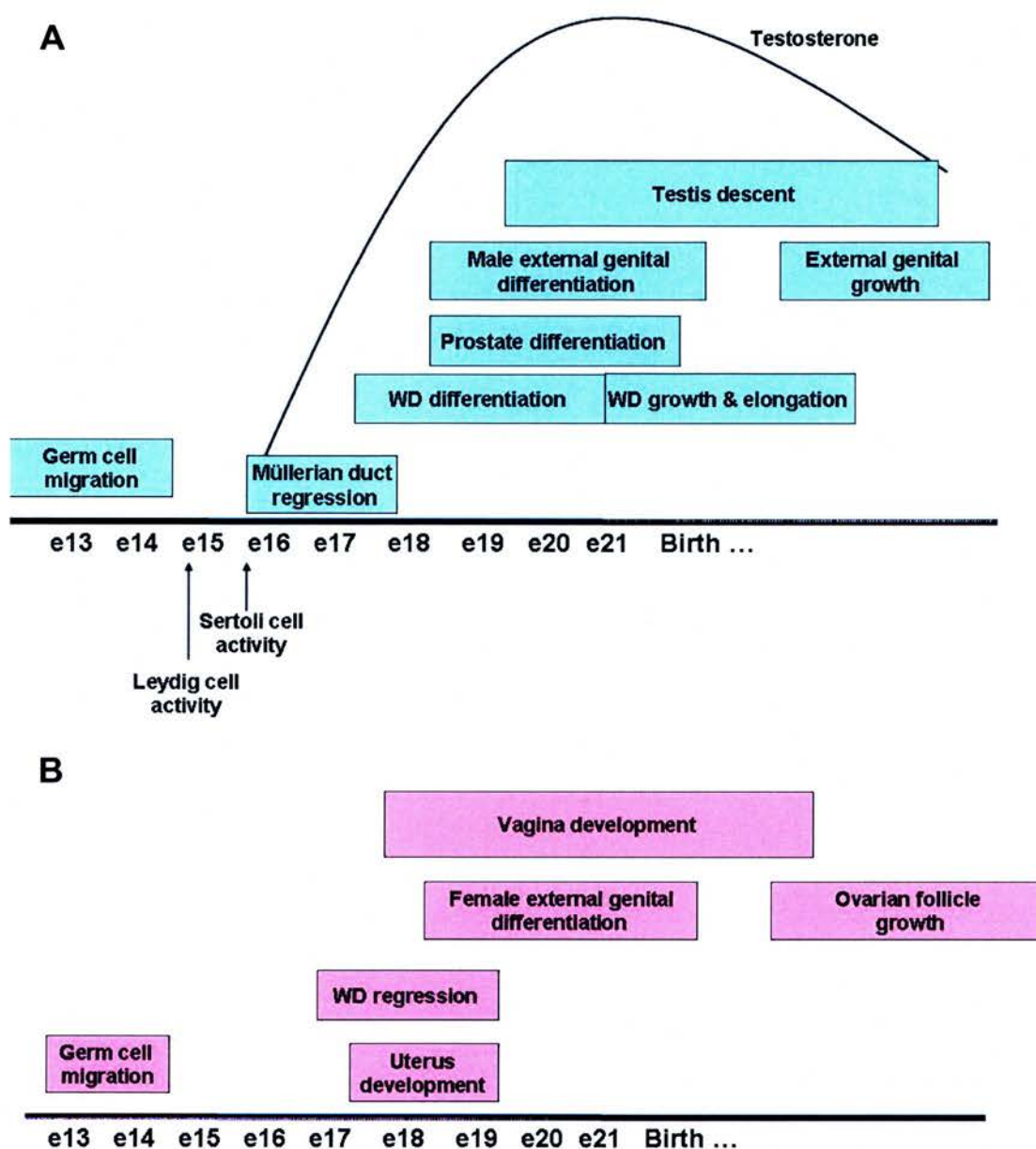


Figure 1.1 Timings of development of male (A) and female (B) reproductive tract in the fetal rat (adapted from Wilson *et al*, 1981; and Hughes, 2001).

1.2 Gonadal development

The indifferent gonads form in males and females from a thickening of the medial side of the genital ridge, which initially developed from the ventrolateral surface of the embryonic mesoderm. The signals which initiate and regulate this thickening are poorly understood but several gene pathways have been suggested to be involved including WT-1, SF-1 and IGF-1R (summarised in Fig. 1.2) (reviewed in Rey and Picard, 1998; Achermann *et al*, 1999; Swain and Lovell-Badge, 1999; Capel, 2000; Brennan and Capel, 2004; Barsoum and Yao, 2006).

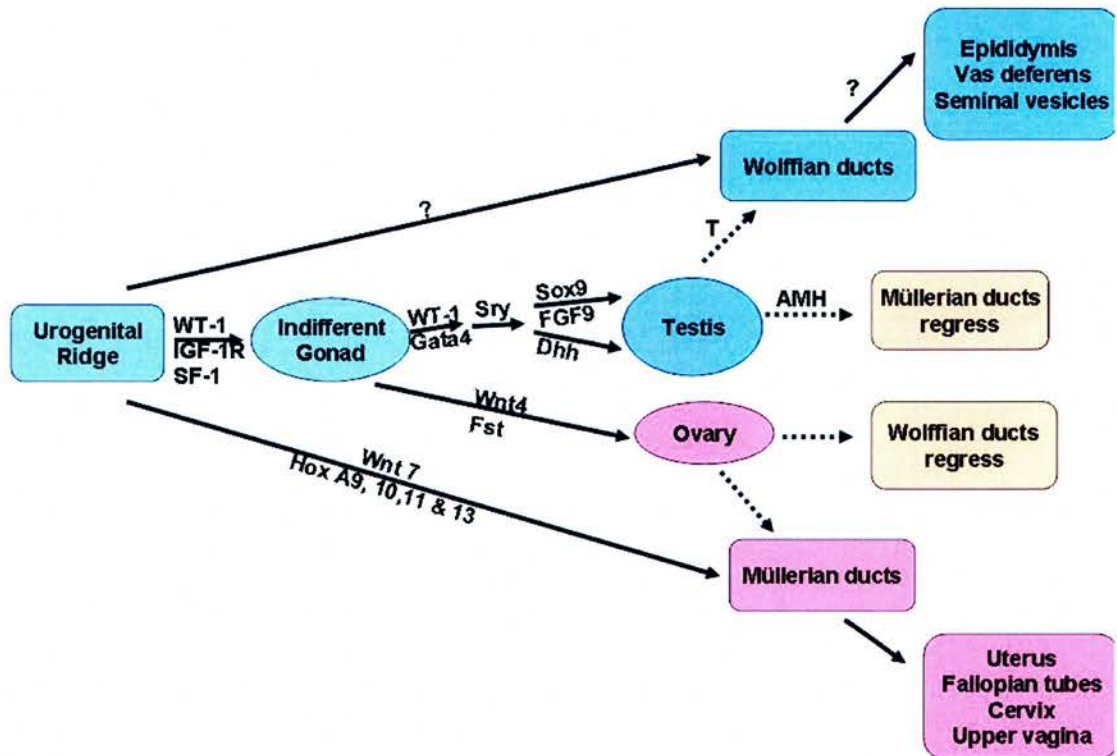


Figure 1.2 Summary of genes and pathways involved in sexual differentiation of the fetal reproductive tract. Several genes are involved in the formation of the bipotential gonad from the urogenital ridge including SF-1, WT-1 and IGF-1R. Gata4 and WT-1 are then required to activate Sry expression in the XY gonad which initiates testis formation; Sox9, FGF9 and Dhh are involved in this testis formation. Conversely, few genes have been characterised in ovary formation except Wnt4 and Fst.

AMH, Anti-Müllerian hormone; Dhh, desert hedgehog; FGF9, fibroblast growth factor 9; Fst, follistatin; Gata4, GATA binding protein 4; Hox A9-13, homeobox transcription factors A9-13; IGF-1R, insulin-like growth factor 1 receptor; SF-1, nuclear receptor subfamily 5; Sox9, Sry-like HMG-box protein 9; T, Testosterone; Wnt 4, wingless-related MMTV integration site 4; Wnt7, wingless-related MMTV integration site 7; WT-1, Wilms tumour homologue. (adapted from Brennan and Capel, 2004)

This bipotential gonad can be identified histologically in the mouse by e9.5 (Kaufman, 1992) and is initially composed of somatic cells, which have migrated from the mesonephros, and primordial germ cells, which have migrated into the genital ridge from the extraembryonic mesoderm in the yolk sac (Ginsburg *et al*, 1990). It is believed that, in eutherian mammals, this process of differentiation of the genital ridge into the testis is driven by active genetic mechanisms and critically depends on the presence of a Y chromosome, whereas the absence of this genetic activity results in ovary formation (Welshons and Russell, 1959). Ovarian differentiation occurs later in fetal life and is independent of the

number of X chromosomes, thus it was initially thought of as the default pathway (Mittwoch, 1969).

1.2.1 Testis development

It has been shown that male sexual determination is due to the sex determining gene, Sry, on the Y chromosome, which is both vital and sufficient to initiate male development (Gubbay *et al*, 1990; Koopman *et al*, 1991). This vital role can be seen in XY mice lacking a functional Sry gene which develop ovaries whereas addition of the Sry gene to XX mice results in testis formation (Gubbay *et al*, 1990; Lovell-Badge and Robertson, 1990; Koopman *et al*, 1991). Furthermore, approximately 10-20% of complete sex reversal patients carry Sry mutations (Hawkins *et al*, 1992). Sry acts as a transcription factor but as yet, its targets and the mechanisms involved remain unclear. It has been suggested that Sry acts in the male to repress a negative regulator which inhibits testis development (McElreavey *et al*, 1993). This negative regulator has been identified as Dax1 (Guo *et al*, 1996). Deletion of this gene can prevent the repressor effect on the masculinising genes thus resulting in testis formation in XX individuals (Guo *et al*, 1996).

The most critical function of Sry is initiation of Sertoli cell differentiation. It is believed that Sry induces Sertoli cell differentiation by regulating another transcription factor, Sry-like HMG-box protein 9 (Sox 9) since Sox9 alone can induce testis formation in mice while deleting Sox9 prevents testis formation in XY mice (Kanai and Koopman, 1999; Qin and Bishop, 2005; Barrionuevo *et al*, 2006). Sox9 is not the only factor involved in Sertoli cell differentiation, however, as Prostaglandin D2 (PGD2), wingless-related MMTV integration site 4 (Wnt4), fibroblast growth factor 9 (Fgf9) and Gata 4 have also all been suggested to play a role, possibly acting parallel to or downstream of Sox9 (Lim and Hawkins, 1998; Nachtigal *et al*, 1998; reviewed in Brennan and Capel, 2004; Barsoum and Yao, 2006) (summarised in Fig. 1.2). Sertoli cell differentiation is a critical event in male sexual development since these somatic cells are responsible for producing factors that influence the further differentiation of the testis as well as the differentiation of the internal and external sex structures (Burgoyne, 1988). In fact, the initiation of the male pathway depends on the development of a threshold number of Sertoli cells (reviewed in Brennan and Capel, 2004). In the mouse, male and female gonads remain morphologically indistinguishable until embryonic day (e)11.5-12.5 at which time the mesenchymal cells in the male aggregate and condense and the pre-Sertoli cells align to form early testicular cords, enveloping the large meiotically inactive germ cells (Bogan and Page, 1994) (reviewed in Brennan, 2004). A basal lamina then forms around these early cords to separate them from the interstitial

mesenchyme. Migration of cells from the mesonephros into the XY gonad is vital in cord formation as blocking this event by culturing the gonad in the absence of a mesonephros impairs cord formation (Buehr *et al*, 1993). This migration depends on growth factors expressed by the Sertoli cells including FGFs, nerve growth factor (NGF) and platelet derived growth factor (PDGF) (reviewed in Brennan and Capel, 2004). This organisation into 2 compartments is vital to provide an environment for germ cell maturation as well as to produce masculinising hormones.

Another key event in testis formation is the development of the testis-specific vasculature. The initial growth of the vasculature from the mesonephros into the gonad is similar in XX and XY gonads but subsequently, following Sry expression, endothelial cells are recruited into the XY gonad to establish the coelomic vessel (Brennan *et al*, 2002). This arterial system elaborates to increase blood flow through the early testis to promote testosterone transport (Brennan *et al*, 2002). It has been proposed that the vasculature may play an active role in further development of the testis and may also be involved in Leydig cell differentiation, however the events in testis development occur rapidly in the mouse and thus make it difficult to establish the relative timings and regulatory events (reviewed in Brennan and Capel, 2004).

Steroidogenic Leydig cells differentiate from the interstitial mesenchyme around 24h after Sertoli cell differentiation in the mouse (Habert *et al*, 2001). This is believed to be regulated by paracrine factors from the Sertoli cells, namely desert hedgehog (Dhh) (Yao *et al*, 2002) and PDGF (Brennan *et al*, 2003), as well as SF-1 and aristaless-related homeobox gene (ARX) expression (Kitamura *et al*, 2002; Park *et al*, 2005). This has been extensively reviewed in the literature and will not be further discussed here (Habert *et al*, 2001; Brennan and Capel, 2004; Haider, 2004; Barsoum and Yao, 2006). Fetal Leydig cells begin to secrete testosterone around e13-14 in the mouse (Pointis *et al*, 1980), e15 in the rat (Inomata *et al*, 1989); Sertoli cells subsequently begin to produce Anti-Müllerian Hormone (AMH), at around e14-15 in the mouse (Munsterberg and Lovell-Badge, 1991). Both AMH and testosterone are vital to drive subsequent male differentiation as detailed below.

The gonads are initially located on the lateral edge of the kidney and following differentiation into a testis, the gonads descend through the abdomen and inguinal canal to reside in the scrotum. This process is believed to be biphasic involving both androgens and other factors including *Ins13*; details of this mechanism are beyond the scope of this review but have been extensively reviewed by other authors (Hutson *et al*, 1997; Nef and Parada, 2000; Nef *et al*, 2000). Contrary to the testis, the ovary does not descend but is positioned close to the kidney throughout fetal life.

1.2.2 Ovary development

Unlike the testis, histological differentiation of the ovary occurs after phenotypic development of the female reproductive tract has been initiated. Initially it was believed that differentiation of the female gonad and reproductive tract occurred by default and did not rely on any active gene regulatory events. However, more recently, this belief has been brought into question (summarised in Fig. 1.2). For example, Wnt 4 has been shown to be vital for gonadal formation and remains expressed in the fetal ovary after sexual differentiation has been initiated to act as an anti-testis gene by repressing aspects of male development in the XX gonad; conversely, expression is lost in the testis (Staack *et al*, 2003). The role for Wnt 4 is further highlighted in the Wnt 4 knockout (Wnt4^{-/-}) mouse which has abnormal ovarian development (Vainio *et al*, 1999). Wnt4 may act by upregulating Dax1, an Sry repressor (Swain *et al*, 1998), thus preventing testis development and resulting in ovary development (Jordan *et al*, 2001); increased Wnt4 and/or Dax1 expression has been implicated to be involved in XY sex reversal (Bardoni *et al*, 1994; Jordan *et al*, 2001). These Wnt4^{-/-} mice also highlighted a role for other female specific genes in ovarian development, namely BMP2 and follistatin, which may act downstream of Wnt4 (Menke and Page, 2002; Yao *et al*, 2004). The masculinisation effects observed in the female Wnt4^{-/-} mice are further discussed in section 1.5.3.2.

During fetal life, the ovary remains a relatively small organ and is not believed to be vital for female urogenital tract development (reviewed in Staack *et al*, 2003). Ovarian primordial follicles normally form prior to birth consisting of germ cells surrounded by supporting granulosa cells; during fetal life however, there is considerable loss of female germ cells which continues postnatally (human, Baker, 1963; mouse, reviewed in De Felici *et al*, 2005).

1.3 Reproductive tract development and differentiation

Unlike gonadal sex, which is primarily determined genetically, sexual differentiation of the reproductive tract depends wholly on hormonal regulation. However, it is not just the secretion of these key hormones which is vital for normal sexual differentiation, but also the expression of their appropriate receptors in the target tissues.

The mammalian reproductive tract is composed of the gonads, the internal genital system and the external genitalia, as summarised in Fig. 1.3. Much of the urogenital system forms from the intermediate mesoderm, which develops during early embryonic development from the intra-embryonic mesoderm (Staack *et al*, 2003). The urogenital ridges can be identified on the posterior wall of the intermediate mesoderm by e9.5 in mice, and within this ridge three overlapping kidney structures develop: the pronephros, mesonephros and metanephros (Staack *et al*, 2003).

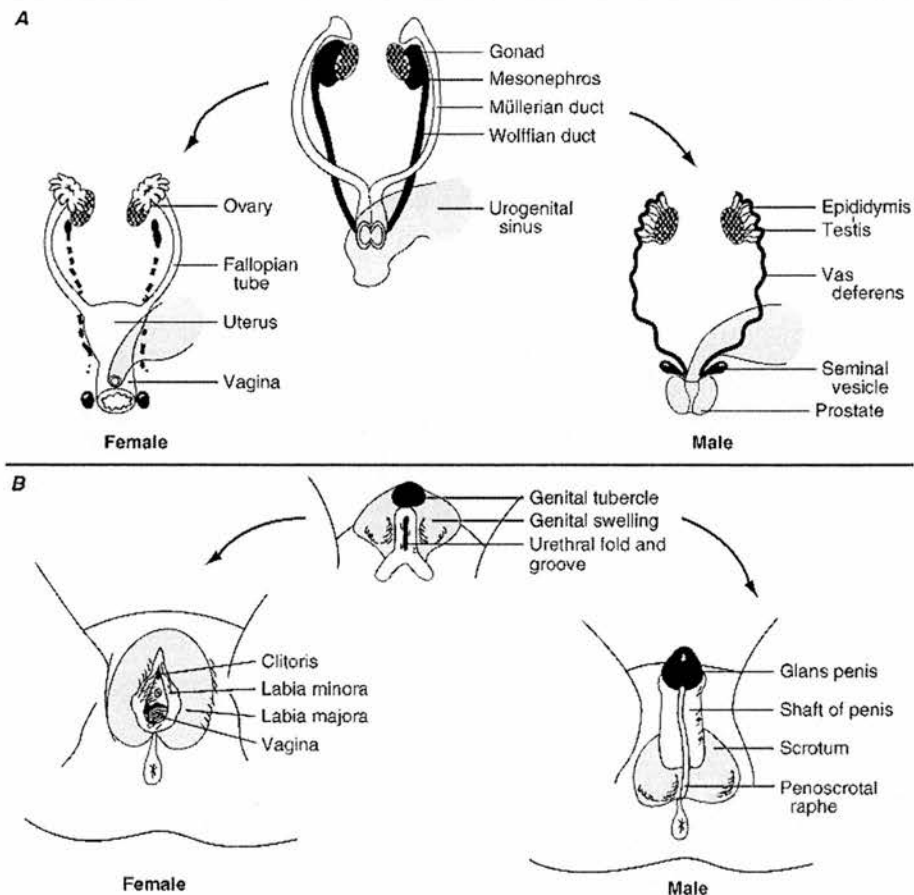


Figure 1.3 Schematic representation of fetal internal (A) and external (B) genitalia (Gilbert, 2003).

1.3.1 Internal genitalia differentiation

The internal genitalia develop from two pairs of ducts, the Müllerian duct (MD) and the Wolffian duct (WD), both of which are derived from the part of the intermediate mesoderm that does not contribute to the gonad. Initially, both sets of ducts are present in both male and female embryos. The pronephros, a transient structure that never functions as an excretory organ, gives rise to a pronephric duct which terminates at the cloaca. The rudimentary pronephros regresses early in embryonic development but the duct persists to form the mesonephric or Wolffian duct by e10 in mice, prior to gonad formation (George, 1994; Staack *et al*, 2003). The cranial end of the WD is connected to the testis via some of the mesonephric tubules which are retained to form the efferent ducts. The Müllerian or paramesonephric ducts are evident later around e12 in mice and form as invaginations of coelomic epithelium on the surface of the urogenital ridge (Staack *et al*, 2003). The MDs lie on the lateral side of the cranial WD but cross to the medial surface of the WD at the caudal end. Just cranial to the UGS, the left and right MDs fuse by e16 in the mouse (Staack *et al*, 2003). Jost (1972) demonstrated that masculinisation of the male reproductive tract depends

on secretions from the fetal testis, unlike the female tract which can differentiate even in the absence of an ovary. Many researchers have subsequently built on this early finding to show that these testicular secretions are in fact, testosterone and Anti-Müllerian hormone (AMH). Unlike the internal genitalia, the urogenital sinus (UGS) is derived from the hindgut as an endodermal tube. The hindgut initially terminates as the cloaca, which subsequently opens to the perineum at the level of the urogenital orifice at the base of the genital tubercle. The cloaca is the terminal part of the embryonic hindgut, which becomes divided by the urorectal septum into the UGS, rectum and anal canal. The UGS is then further subdivided to form the bladder and the definitive UGS by e13-14 in the mouse with the MD and WD terminating in this UGS (Staack *et al*, 2003).

1.3.1.1 Male

Masculinisation of the male reproductive tract is initiated following differentiation of Sertoli cells and organisation of seminiferous cords in the testis (Wilson *et al*, 1981). The first step is the sexually dimorphic process of MD and WD differentiation, which begins around e15 in the mouse, e16 in the rat (Inomata *et al*, 1989). In the male, the MD begins to regress in a cranio-caudal direction at e15 in the mouse, e16 in the rat (Inomata *et al*, 1989; Staack *et al*, 2003). This process is mediated by AMH, which is secreted by the fetal Sertoli cells and binds to a specific receptor (AMHR2) expressed in the mesenchyme around the MD (Donahoe *et al*, 1977; Roberts *et al*, 1999; Allard *et al*, 2000). The actions of AMH extends beyond the scope of this review but has been extensively discussed in previous literature (Donahoe *et al*, 1977; Wilson *et al*, 1981; Josso *et al*, 2001; Roberts *et al*, 2002; Xavier and Allard, 2003). MD regression can be characterised by disruption of the basement membrane allowing direct contact between the epithelium and mesenchyme, thus leading to a decrease in epithelial cell size and subsequent epithelial degeneration (Dyche, 1979; Ikawa *et al*, 1984; Inomata *et al*, 1989; Austin, 1995). This regression is thought to be driven by paracrine mechanisms originating in the mesenchyme as a result of a loss of extracellular matrix (ECM), and they induce the formation of an 'epithelial cuff' due to condensation of the periductal mesenchyme (Dyche, 1979; Inomata *et al*, 1989; Austin, 1995). In mammals, it is unclear whether the mesenchyme is also lost during regression, as occurs in alligators (Austin, 1995). Apoptosis is evident in the regressing MD, probably stimulated by the loss of cell contact with the basement membrane and ECM, but is not believed to be sufficient to cause complete regression of the male MD (Allard *et al*, 2000; Xavier and Allard, 2003). Some epithelial cells remain viable in the regressing MD and are believed to dedifferentiate into mesenchymal-like cells (Dyche, 1979; Jirsova and Vernerova, 1993; Roberts *et al*, 1999;

Allard *et al*, 2000), possibly even migrating to contribute to the mesonephros in some species (Hutson *et al*, 1997).

After the onset of MD regression, testosterone synthesised by the Leydig cells that differentiate within the interstitium of the fetal testis stabilises and differentiates the male WD (Dyche, 1979); this process begins around e14 in the mouse (Staack *et al*, 2003) and e16 in the rat (Inomata *et al*, 1989). Testosterone is transported from the testis down the WD lumen to ensure delivery of high levels of androgen directly to the site of action (Tong *et al*, 1996); it has been postulated that this may occur by passive diffusion or actively transported from the testis to the WD however, the exact mechanism is not yet understood. Once rescued, the cranial segment of the WD elongates and convolutes to form the epididymis, the middle portion remains a straight simple tube to form the vas deferens, and the caudal tip buds to form the seminal vesicles (George, 1994; Staack *et al*, 2003). This process begins around e18 in the mouse and continues throughout neonatal life. Each of these WD derived structures can be identified by their differing morphology, gene expression and function. The future epididymis, the site of sperm maturation in the adult, becomes surrounded by a concentric layer of mesenchymal cells which ultimately differentiate into smooth muscle while the vas deferens is surrounded by 3 layers of smooth muscle and transports mature sperm to the urethra for ejaculation (George, 1994). The caudal segment of the WD distends and elongates and the lumen becomes complexly folded in order to form the seminal vesicles at around e17 in the mouse (George, 1994; Staack *et al*, 2003), and e18 in the rat (Inomata *et al*, 1989); these vesicles produce seminal or ejaculatory fluid in the adult. The mechanisms involved in seminal vesicle development are beyond the scope of this review but are further discussed by Marker and colleagues (2003). The differentiation of the WD into these adult-derivatives is dependent on testosterone action and is vital for the maturation and transport of sperm in the adult from the testis out of the body, but little is known about the mechanisms acting downstream of androgen action or the processes involved in patterning the development of these three very different adult derivatives of the WD (Jost *et al*, 1973). Considerable research has been undertaken to investigate the role for stromal-epithelial interactions, patterning genes and growth hormones in male sexual differentiation, as discussed in section 1.8.

The prostate develops as branching and budding of the UGS at around e17 in the male mouse (Staack *et al*, 2003), and e18-19 in the rat (Inomata *et al*, 1989). The prostate is originally made up of two portions, each of which develops as a series of buds from the epithelium lining the UGS and cloaca (Gray, 1918). These buds become tubular and meet and fuse behind the urethra to form the glandular structures of the 2 main prostatic lobes.

Prostate development has been extensively studied and reviewed in the literature and will not be discussed in detail here (Cunha *et al*, 1987; Cunha *et al*, 1996; Thomson *et al*, 2002; Marker *et al*, 2003; Thomson and Marker, 2006).

1.3.1.2 Female

Unlike the male, the female duct system does not depend on hormonal action to initiate or control its differentiation. Oestrogen synthesis is temporarily activated in rabbit and human male and female embryos prior to implantation and this ability to synthesise oestrogens from androgen continues in the ovary but is minimal in the fetal testis (George and Wilson, 1978). In rabbits, this oestrogen synthesis begins in the ovary prior to histological differentiation, around the same time as testosterone synthesis onset in the testis (Milewich *et al*, 1977; George and Wilson, 1978). This could suggest a possible role for oestrogens in female development, however, subsequent studies have shown that oestrogen is not vital for female reproductive tract differentiation (reviewed in Couse and Korach, 1999). The female MD is programmed to differentiate into the oviducts, uterus, cervix and upper vagina. However, it is interesting to note that the female MD expresses the AMHR and exposure to exogenous AMH can induce MD regression in females (Teixeira and Donahoe, 1996). Further details on female MD differentiation have been extensively reviewed in the literature (George, 1994; Du and Taylor, 2004; MacLaughlin and Donahoe, 2004; Yin and Ma, 2005). Briefly, the MD epithelium adopts organ-specific cell fates along its length to form each of the adult derivatives (Kurita *et al*, 2001; Yin *et al*, 2006). In some species such as rodents, the mid portion of the MDs remain separate to form paired uteri while in others they fuse, as in humans; the reason for this difference and the mechanisms involved are not fully understood (George, 1994). Vaginal development is believed to be negatively regulated by androgens since endogenous testosterone in males prevents vaginal formation and results instead in the budding of the caudal tips of the WD to form the seminal vesicles (Bok and Drews, 1983; Drews, 2000). It is accepted that the caudal tips of the female MD contribute to the formation of the vagina but controversy exists over which other structures also contribute; some researchers believe that, in the absence of androgens, prior to complete regression of the WD the caudal tips of the WD link the UGS to the MD (O'Rahilly, 1977; Drews, 2000). As in the male reproductive tract, the morphology and fate of the epithelium in the developing female UGT is dictated by the adjacent mesenchyme (Cunha, 1976) (discussed further in section 1.8). It has been suggested that Wnt signalling is involved in regulating this epithelial-mesenchymal signalling (Sassoon, 1999). Wnt genes are highly expressed during female development and are involved in growth and differentiation (Heikkila *et al*, 2001). Exposing animals perinatally to diethylstilbestrol (DES), a synthetic oestrogen,

permanently alters patterning of the female UGT partly by de-regulating Wnt gene expression during morphogenesis (Newbold and McLachlan, 1982). Studies in Wnt knockout animals have further highlighted the critical role for Wnt genes in female reproductive development (reviewed in Heikkila *et al*, 2001; Bernard and Harley, 2006; Kim *et al*, 2006). For example, the MDs fail to form in both male and female Wnt-4 knockout embryos (Vainio *et al*, 1999). Another gene known to be involved in female UGT development is p63, a homologue of the tumour suppressor gene p53 (Kurita and Cunha, 2001; Ince *et al*, 2002). p63 knockout female mice have abnormal genital morphogenesis with hypoplastic genitalia and a lack of urothelial differentiation (Ince *et al*, 2002). These mice highlight a role for p63 in determining the identity switch for cell fate in the MD since, in the absence of p63, the cervicovaginal epithelium wrongly differentiates to a more uterine-like epithelium (Kurita *et al*, 2004). It is believed that p63 plays a role in differentiating the basal regenerative cells and influences the modelling of the UGT by altering proliferation, apoptosis and cell cycle arrest (Ince *et al*, 2002).

Due to the lack of androgens, the female WD begins to regress in a cranio-caudal direction. In the rat, the WD appears morphologically and histologically similar at e15-16 but has almost completely regressed by e19 (Jirsova and Vernerova, 1993). In the mouse these events occur at e14-15 and e16, respectively (Price, 1965; Dyche, 1979). WD regression can be identified histologically by the reduction in diameter of the duct and lumen as well as degeneration of the epithelial lining (Inomata *et al*, 1989; Jirsova and Vernerova, 1993). It has been suggested that the epithelium loses its polarity and the epithelial-mesenchymal boundary becomes irregular. Previous researchers have reported that apoptosis is involved in female WD regression, but that some non-altered epithelial cells can still be identified late in WD regression suggesting a possible role for epithelial-mesenchymal transformation (Jirsova and Vernerova, 1993). However, prior to complete regression of the WD, the caudal segment of the WD contributes to the formation of vaginal plate, as detailed above (Bok and Drews, 1983; George, 1994).

1.3.2 Differentiation of the external genitalia differentiation

Unlike the internal genital ducts which develop from different ductal systems in males and females, the external genitalia develop in both sexes from common primordial structures, namely the genital tubercle, genital swellings and urethral folds (Wilson *et al*, 1981) (summarised in Fig. 1.3). Once again, the development of the male phallus requires a hormonal stimulus unlike the female external genitalia which develop autonomously without any direct hormonal influence (MacLaughlin *et al*, 2001).

1.3.2.1 Male

After the onset of masculinisation of the internal duct system, the genital tubercle elongates to form the body of the penis, the urethral folds elongate and fuse to form the penile urethra while the urogenital swellings fuse to form the scrotum, which will ultimately house the testes outside the body cavity (Wilson *et al*, 1981; Yamada, 2003). This is dependent on the conversion of testosterone to the metabolically active dihydrotestosterone (DHT) by the enzyme 5-reductase (Wilson *et al*, 1981). Hypospadias results when the urethra is not completely enclosed by the urethral folds with the site of fusion failure dictating the position of the abnormal opening of the urethra (reviewed in Yamada, 2003). In rodents, the male external genitalia do not undergo significant differential growth so that by birth, the phallus size does not differ greatly between males and females (Wilson *et al*, 1981). Conversely, in humans sex can easily be determined externally at birth. However, in both rodents and humans, the distance between the anus and the base of the genital tubercle (anogenital distance, AGD) increases in males in response to androgens thus at birth AGD is often used as a measure of fetal androgenisation (reviewed in Swan *et al*, 2005; reviewed in Edwards *et al*, 2006).

1.3.2.2 Female

Differentiation of the female external genitalia begins later than in the male with little differentiation occurring during fetal life in rodents (Wilson *et al*, 1981; reviewed in Yamada, 2003). The genital tubercle differentiates into the clitoris while the genital swellings give rise to the labia majora and the folds to the labia minora (Wilson *et al*, 1981). Contrastingly, in humans the female external genitalia undergo considerable differentiation by 26 weeks gestation (Ammini *et al*, 1994).

1.4 Steroidogenesis

Steroid hormones are vital for the normal development and differentiation of the fetal reproductive tract; in particular the androgen, testosterone, is essential for normal male development. The pathway and enzymes involved in testosterone biosynthesis in the fetal testis have been extensively studied and reviewed in the literature (Sharpe, 1990; Huhtaniemi and Toppari, 1995; Stocco and Clark, 1996; Miller, 1998) and will only be briefly discussed here. The main enzymatic steps in steroidogenesis are outlined in Fig. 1.4. One of the key steps is the rate limiting conversion of cholesterol into pregnenolone and the subsequent formation of DHEA. This step is controlled by the steroidogenic acute regulatory protein (StAR) which transfers cholesterol from the outer to the inner mitochondrial membrane, and two enzymes namely p450 side chain cleavage (P450scc), which cleaves the side chain from this cholesterol, and p450c17, which converts pregnenolone into DHEA (Miller, 1998;

Handelsman, 2003). Many of these early enzymatic reactions are common to the production of glucocorticoids, mineralocorticoids and sex steroids. Within the gonads, DHEA is converted into testosterone or oestradiol by the actions of 3β -hydroxysteroid dehydrogenase (3β -HSD), aromatase and 17β -HSD.

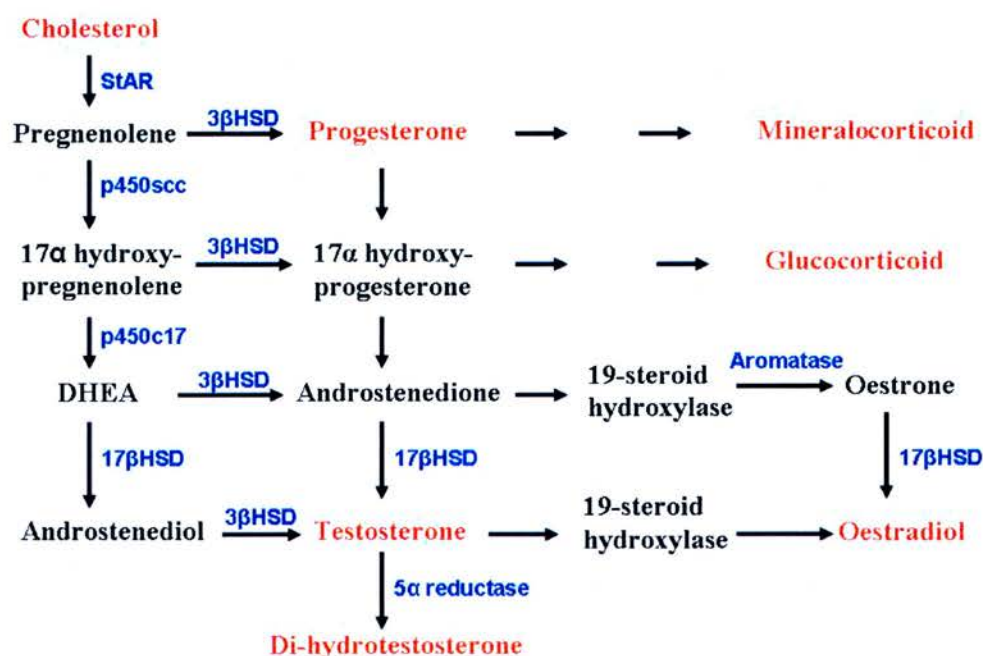


Figure 1.4 Pathway of steroidogenesis. The key stages in steroidogenesis are highlighted in red while the key enzymes are coloured blue (Adapted from Holmes *et al*, 2004).

1.4.1 Testicular testosterone biosynthesis

Testosterone is the main steroid hormone in the male reproductive tract, both during fetal life and postnatally. Testosterone biosynthesis takes place in the fetal and postnatal testis in the Leydig cells, with its further metabolism into oestradiol by aromatase or dihydrotestosterone (DHT) by 5α -reductase occurring mainly in the testes or within the target tissues such as the prostate or seminal vesicles, respectively (Huhtaniemi and Toppari, 1995; Carreau *et al*, 2006). Postnatally, testosterone production is driven by luteinising hormone (LH) which acts via the LH receptor expressed on Leydig cells (Warren *et al*, 1975; Huhtaniemi, 1994; Huhtaniemi and Toppari, 1995; Majdic *et al*, 1998). LH action on the Leydig cells can be modified by various paracrine and autocrine factors including prolactin and growth factors, thus regulating testicular testosterone biosynthesis (reviewed in Huhtaniemi and Toppari, 1995; Hedger and de Kretser, 2000; Haider, 2004; O'Shaughnessy *et al*, 2005). The onset of testicular testosterone biosynthesis occurs at around e15 in rats, almost as soon as the fetal Leydig cells have differentiated and peaks around e18.5 (Warren *et al*, 1972; Tapanainen *et*

al, 1981; Habert and Picon, 1984). In humans, testosterone is produced by the fetal testis around week 7 gestation, approximately 1 week after sexual differentiation of the gonad (reviewed in Habert *et al*, 2001). Testicular testosterone production precedes biosynthesis of pituitary gonadotrophins suggesting that, unlike postnatal testosterone biosynthesis, it is independent of LH stimulation in the fetus, at least initially (El-Gehani *et al*, 1998; Majdic *et al*, 1998; O'Shaughnessy *et al*, 1998; Habert *et al*, 2001); it is presumed that locally-produced factors drive fetal testosterone synthesis but these factors remain to be defined. Studies in fetal rats have shown that plasma testosterone levels are at least 4 times higher in the male than in the female, in which levels remain very low throughout fetal life (Habert and Picon, 1984).

Testosterone is secreted from the fetal testis into the bloodstream, where it binds to sex-hormone binding globulin (SHBG) in humans or albumin in rodents, and reaches structures distal to the testis at relatively low concentrations (reviewed in Grishkovskaya *et al*, 2000; reviewed in Handelsman, 2003). Alternatively, testosterone can be transported, via unknown mechanisms, directly into the WD lumen to reach the cells of the developing WD in relatively high concentrations, especially at the cranial end (Tong *et al*, 1996). Once released from the testis, testosterone can be further metabolised in target tissues into the more potent androgen, DHT by the enzyme 5 α -reductase (Fig.1.5) (Jaffe, 1969; Wilson and Lasnitzki, 1971; Cooke and Robaire, 1987; Russell and Wilson, 1994; Tsuji *et al*, 1994; Berman *et al*, 1995). Two isoforms of 5 α -reductase exist, type I which is expressed in the liver and skin, and type II which is expressed in the UGT and liver (Thigpen *et al*, 1993). In the fetal male, DHT metabolism occurs in peripheral tissues, rather than in the testis where DHT levels are barely detectable (Habert and Picon, 1984). Expression of 5 α -reductase in different tissues is further reviewed in section 1.6.1.2.

Studying patients with androgen biosynthesis deficiencies can provide a great deal of insight into the role of androgens in masculinisation (Zhu *et al*, 1998; Brinkmann, 2001; Hannema *et al*, 2004; Hannema *et al*, 2006). It is interesting to note that the variation in phenotypes observed in these patients depends upon the cause of the deficiency. For example, patients with a deficiency in 5 α -reductase type II have normal WD development but lack masculinisation of the external genitalia at birth (Imperato-McGinley *et al*, 1974; Brinkmann, 2001; Imperato-McGinley, 2002) whereas XY male patients with AR mutations often completely lack any male reproductive tissue other than testes (Hughes and Evans, 1987). This highlights the differential roles for testosterone and DHT in different tissues and the dependence of complete masculinisation on local amplification of androgen action by 5 α -reductase expression.

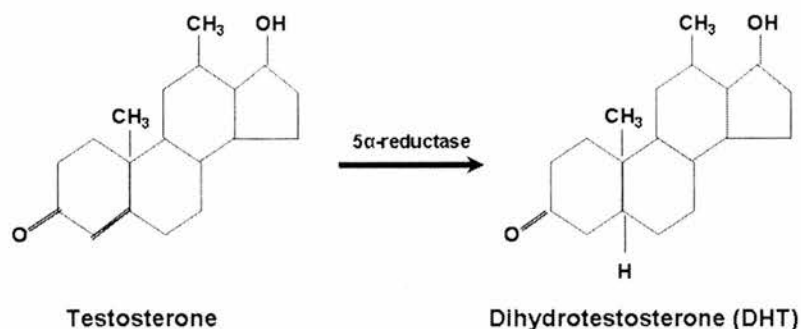


Figure 1.5 Structure of testosterone and dihydrotestosterone (DHT) (adapted from Brinkmann, 2003).

1.4.2 Steroid Receptors

Steroid hormones can enter almost any cell in the body, but only some cells are capable of binding the steroid so eliciting a response and therefore altering gene expression; this is due to the presence of intracellular steroid receptors which are members of a large superfamily of ligand-activated transcription factors (Evans, 1988; Parker, 1990; Mangelsdorf *et al*, 1995; Couse and Korach, 1999; Gelmann, 2002). The androgen receptor (AR) is a member of the steroid receptor family which also includes the glucocorticoid, progesterone and oestrogen receptors; each of these receptors is structurally and functionally similar (reviewed in Gobinet *et al*, 2002).

1.4.2.1 Steroid receptor structure

Steroid hormone receptors share a common structure and are made up of 4 main functional domains; the DNA-binding, ligand-binding, hinge and variable domains (Fig. 1.6). All steroid receptors have a DNA-binding domain which is highly conserved and contains two zinc fingers (reviewed in Carson-Jurica *et al*, 1990; Mangelsdorf *et al*, 1995). The C-terminal zinc finger is believed to be involved in protein-protein interactions which are vital in transcriptional activity of the nuclear receptor (reviewed in Freedman, 1992). Studies in patients with a mutation in exon C of the AR, which encodes the second zinc finger, highlight the vital role for this zinc finger in DNA-binding since the AR in these individuals had a reduced ability to bind to DNA; this finding offers some explanation for the occurrence of complete androgen insensitivity in patients in which the AR is capable of androgen binding (Quigley *et al*, 1992). Between the two zinc fingers is an amino acid sequence which can recognise the specific DNA response element thus making this domain vital for subsequent DNA transcription (Beato *et al*, 1996). The ligand binding domain is less conserved and is situated at the C terminus; it determines ligand specificity as well as binding heat shock proteins (reviewed in Carson-Jurica *et al*, 1990; Mangelsdorf *et al*, 1995).

The hinge domain lies between the ligand- and DNA-binding domains and controls the movement of the receptor into the nucleus as well as modulation of transcriptional activation (reviewed in Carson-Jurica *et al*, 1990; Mangelsdorf *et al*, 1995). As the name suggests, the variable domain at the N-terminus is the least conserved among the nuclear receptors and its function has not yet been defined; suggestions include a role in interactions with other domains of the receptor to promote complete transcriptional activation or a role in ligand-independent transactivation function (Simental *et al*, 1991).

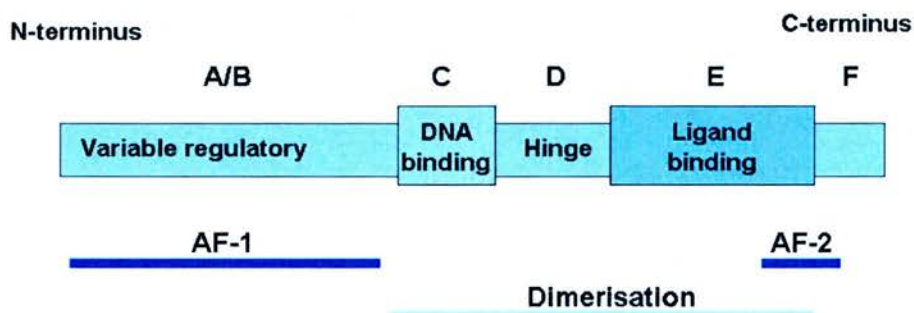


Figure 1.6 Basic structure of steroid receptors

1.4.3 Androgen receptor

Androgens are believed to be able to act through both genomic (direct) and non-genomic (indirect) mechanisms but it has been thought that the majority of their action is through direct activation of DNA transcription via high affinity interactions with intracellular AR expressed in target tissues (Verhoeven and Swinnen, 1999). The AR resides in the cytoplasm and translocates to the nucleus upon hormonal stimulation. In the rat, withdrawal of this hormonal stimulation by castration, results in the AR relocating back to the cytoplasm in the prostate within just 1 day (Van Doorn and Bruchovsky, 1978). The AR is coded for by a single copy gene containing 8 coding exons, which is found on the X chromosome at Xq11-12 in humans (Brown *et al*, 1989). AR mRNA normally codes for a 919 amino acid protein (110-144 kDa) but reports exist of a second smaller AR protein (87kDa) which arises due to alternative splicing in the 3'-untranslated region and only contributes to a very small proportion of the total AR detected in tissues; the relevance of this second AR is unknown (Faber *et al*, 1991; Wilson and McPhaul, 1996). The rat AR shows 83% nucleotide homology and 85% amino acid homology with the human AR (Chang *et al*, 1988; Tan *et al*, 1988).

The AR has an activation function (AF-1) region in the N-terminus variable domain, AF-2 in the ligand binding domain at the C-terminus, and a polymorphic glutamine region with a variable number of CAG repeats; variation in the number of repeats is believed to alter the transcriptional efficiency of the AR (Chamberlain *et al*, 1994). Androgen dependent gene

transcription is regulated by interactions between the AF domains of the AR and co-regulator proteins, which can act to either increase or decrease transcription (McKenna *et al*, 1999; McEwan, 2004). These include steroid receptor coactivator 1 (SRC-1), CREB-binding protein (CBP), transcriptional intermediary factor 2 (TIF-2), and steroid receptor RNA activator (SRA) (Onate *et al*, 1995; Berrevoets *et al*, 1998; Bevan *et al*, 1999).

To date, hundreds of mutations have been identified in the human AR, with the mutations occurring throughout the gene. Initially, the majority of these mutations were identified in the steroid binding domain but this was probably due to a bias selection towards patients with complete androgen insensitivity rather than an increased prevalence of mutations in this region (Hiort and Holterhus, 2000). The AR mutations vary from point mutations to deletions of entire exons (Quigley *et al*, 1992) and result in varying degrees of androgen insensitivity including complete inactivation of the AR, presenting clinically as a genetic male but with no signs of genital masculinisation (Quigley *et al*, 1992), mutations which impair AR mRNA stability (Choong *et al*, 1996) or over-elongation of the polyglutamide trinucleotide repeat region resulting in mild androgen insensitivity presenting as impaired spermatogenesis (Tut *et al*, 1997). These are discussed further in section 1.6.2.2.

1.4.3.1 Mechanism of AR action

Testosterone and DHT bind to the same androgen receptor (AR) but with varying affinity; testosterone has a two-fold lower affinity for AR than does DHT, and dissociates from the AR five times faster than does DHT. Unliganded AR is bound to heat shock proteins (HSP) in the cytoplasm, which stabilise the conformation of the ligand-binding domain and prevents the receptor associating with its specific hormone response element on the DNA (Picard *et al*, 1990; Jenster *et al*, 1993). Upon ligand binding the HSP is shed and the ligand-receptor complex is transported into the nucleus (Jenster *et al*, 1993). The AR is then phosphorylated; this allows specific regulation of gene expression by cell specific kinases (van Laar *et al*, 1990; Orti *et al*, 1992; Kuiper *et al*, 1993). Steroid receptors do not possess intrinsic protein kinase activity however, hormone exposure increases steroid receptor phosphorylation which has been shown to be important in the acquisition of ligand-binding activity (Golsteyn *et al*, 1989; Hoeck *et al*, 1989; Migliaccio *et al*, 1989; Orti *et al*, 1989). The unliganded AR is constitutively phosphorylated on serine residue 94 but upon ligand binding, the AR becomes hyperphosphorylated (Gioeli *et al*, 2002). This hormone-dependent phosphorylation must occur before or during AR conformational change, but not after (van Laar *et al*, 1991). It has been proposed that over 90% of the hormonally regulated phosphorylation sites in the AR are at the N-terminal transactivation domain and involves at least serine residues 16, 81, 256, 308, 424, and 650 (Kuiper *et al*, 1993; Gioeli *et al*, 2002).

For example, AR can be phosphorylated at Ser-213 by Akt in the presence of androgens but cannot be stimulated by anti-androgens or growth factors in the absence of androgens (Taneja *et al*, 2005). This process was seen to be tightly regulated in a cell type-specific manner (Taneja *et al*, 2005). This phosphorylation of AR at Ser-213 was seen in the epithelium of the human urogenital sinus early in development when androgen levels are high but was absent later in development when androgen levels and Akt activation are lower (Taneja *et al*, 2005). Furthermore, a tyrosine residue on the AR can be phosphorylated by a cAMP-dependent protein kinase (Golsteyn *et al*, 1989).

The AR undergoes a conformational change necessary for translocation; this reveals the DNA-binding domain necessary for interaction with AREs on target genes (Grino *et al*, 1987). The AR-androgen complex must dimerise in order to bind to androgen response elements (AREs) on target genes and thus elicit a transcriptional response (Jones, 1990) (summarised in Fig. 1.7). The consensus binding sequence for the AR is a 15 b.p sequence which comprises two imperfect 6 b.p indirect repeats; this sequence is almost identical to the glucocorticoid response element (Roche *et al*, 1992). The classical ARE does not discriminate between binding of the DNA-binding domain of the AR and glucocorticoid receptor (GR) *in vitro*, however more recently an androgen specific response element has been identified which binds the AR but not the GR therefore conferring androgen-specific transcriptional control (Claessens *et al*, 1996; Schoenmakers *et al*, 1999).

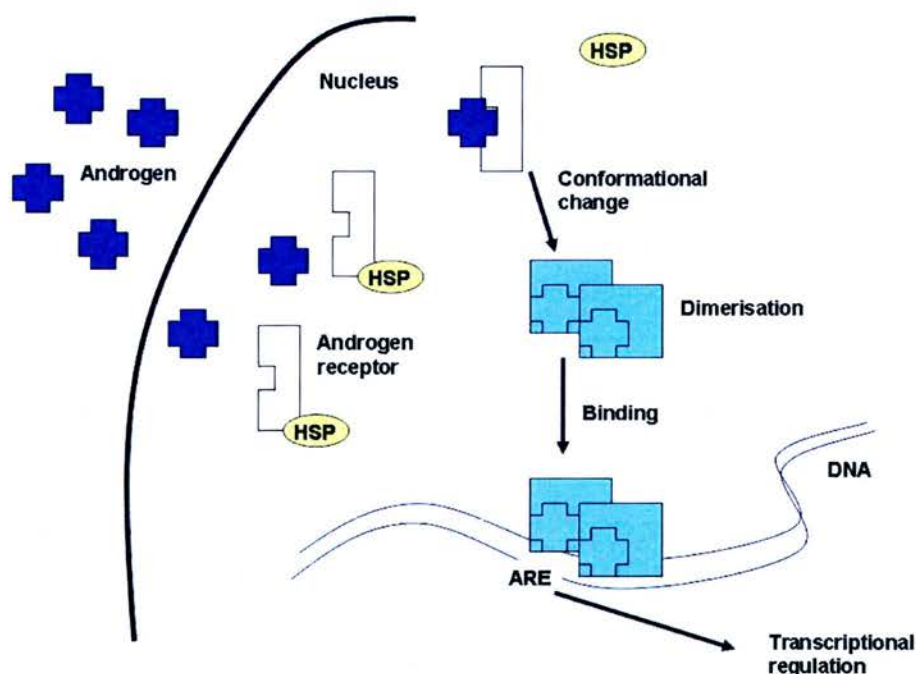


Figure 1.7 Summary of mechanism of androgen receptor action upon ligand binding. Androgen response element, ARE; Heat shock protein, HSP. Adapted from in house figure from Prof. P. Saunders.

Transcriptional regulation of target genes by steroid hormones is complex and involves many steps including hormone binding, receptor phosphorylation at serine residue 94, dissociation of HSP, DNA binding, and activation of transcription, many of which are still not fully understood (Evans, 1988; McEwan, 2004; Wu *et al*, 2005; Kishimoto *et al*, 2006). It is known, however, that transcriptional regulation by androgens is dependent on the presence of co-factors which can interact with the AR to increase or decrease transcription (McKenna *et al*, 1999). It has been proposed that, once transcriptionally activated, the steroid receptors transfer to a common compartment or foci within the nucleus where they interact with co-activators in order to initiate complete transactivation (Saitoh *et al*, 2002). These co-regulators can act at many stages within the AR signalling pathway, for example altering chromatin structure or facilitating N-C terminal interactions and include SRC-1, TIF-2 and GRIP-1 (reviewed in McKenna *et al*, 1999; Rosenfeld and Glass, 2001). Some co-activators can act as histone acetyltransferases, such as SRC-1, thereby relaxing the chromatin packaging thus allowing gene transcription by steroid hormones; on the other hand, co-repressors tend to recruit histone deacetylases, thereby condensing the nucleosome and so repressing gene transcription (reviewed in McKenna *et al*, 1999; Glass and Rosenfeld, 2000). A growing list of AR co-regulators has been identified suggesting that a complex network of factors may be involved in regulating AR transcription (reviewed in Heinlein and Chang, 2002) with many co-regulators that interact with the AR also able to interact with other nuclear receptors (reviewed in McKenna *et al*, 1999; Rosenfeld and Glass, 2001). It remains somewhat of a mystery how androgens signalling through this one AR can induce such different responses in different tissues at different times: co-regulator expression may be cell type-specific, therefore their role in AR-mediated transcription may go some way towards providing insight into this phenomenon. As mentioned above, androgens may also act in a rapid non-genomic way in the absence of functional ARs to indirectly alter the expression of genes that do not contain AREs. The mechanisms behind this phenomenon are not fully understood. However, it is hypothesised that androgens may alter the activity or concentration of secondary transcription factors, hormones or paracrine or autocrine mediators to elicit a response in the absence of ARs (Verhoeven and Swinnen, 1999). For example, growth hormone (GH) exposure can mimic androgen effects in the AR deficient Tfm mouse (Georgatsou *et al*, 1993).

There is also evidence of other nongenomic pathways of androgen action in which intracellular mechanisms mediate their effects, for example activation of Src-Raf1/Shc-Erk2. Androgens and/or oestrogens induce the assembly of a novel ternary complex comprising the AR, ER and Src; this activates the protein kinase domain of Src, resulting in downstream

effects such as cell proliferation (Migliaccio *et al*, 2000). The androgen–AR and estrogen–ER complexes bind to separate domains on the Src protein, which can be blocked by steroid receptor antagonists (Migliaccio *et al*, 2000).

1.4.3.2 Regulation of AR expression

Levels of expression of AR protein are regulated at both the transcriptional and post-transcriptional level. AR is capable not only of regulating transcription of other genes but also of autoactivation of its own gene to make more ARs (Bentvelsen *et al*, 1994; Bentvelsen *et al*, 1995). AR mRNA stability is essential in the regulation of AR protein expression: studies have shown that RNA-binding proteins bind to a highly conserved UC-rich element in the 3' untranslated region of the AR mRNA, which alters AR protein expression (reviewed in Yeap *et al*, 2004). Studies undertaken by Kemppainen have shown that androgens can decrease AR mRNA levels but can still increase AR protein half-life (Kemppainen *et al*, 1992). AR expression is regulated differentially in different tissues and at different stages of development. For example, it has been shown in rats that withdrawal of androgen results in involution of the prostate and reduced prostatic AR levels (Cain *et al*, 1994); testosterone therapy can regenerate the prostate and its AR expression (Prins and Birch, 1993). However, it is worth noting that AR protein stability is enhanced upon ligand binding and thus more AR protein may be detected despite reduced transcription of the mRNA (Bentvelsen *et al*, 1994).

Oestrogens can also regulate AR expression. Exposure of neonatal rats to oestrogens induced a loss of AR immunoexpression in association with abnormalities in the pre-pubertal testes and reproductive tract (Williams *et al*, 2001). However, it should be noted that these adverse effects are due to the combined effect of high oestrogen and low testosterone as neither high oestrogen in the presence of high androgens or low testosterone in the absence of oestrogens could induce these changes (Williams *et al*, 2001). Furthermore, exposure of neonatal rats to oestradiol did not alter AR mRNA expression in the ventral prostate but did permanently decrease AR protein expression (Woodham *et al*, 2003). This reduction in AR protein levels appears to be due to an increase in receptor protein degradation due to increased ubiquitination, rather than due to transcriptional modifications (Woodham *et al*, 2003).

AR expression is regulated post-translationally by ubiquitination and proteasome activity; this process is not just a typical protein degradation event caused by proteasomes but is believed to be more complex (Jaworski, 2006). This mechanism is highlighted in experiments which have shown that AR N-terminal interacting protein (ARNIP) can function as a ubiquitin-protein ligase and reduce AR N-C terminal interactions in Cos cells thereby reducing AR activity (Beitel *et al*, 2002). The role for proteasomes in AR regulation

however is still not fully understood. Previous studies suggest that the ability of the ligand bound AR to recruit polymerase II and co-regulators to the promoter of the androgen responsive gene involves proteasomes (Kang *et al*, 2002). Transcriptional activation by steroid hormones is a cyclical event with AR only transiently binding to the ARE and co-regulators; hormone binding to the steroid receptors increases ubiquitination and degradation of the nuclear receptors and it has been suggested that this degradation of the AR and/or co-regulators is vital for subsequent rounds of transcription to take place (Ferdous *et al*, 2001; Kang *et al*, 2002). This process of degradation has been suggested to be mediated by proteasomes since proteasome inhibitors can suppress AR transactivation in LNCap cells, possibly by inhibiting AR nuclear translocation and/or preventing AR interaction with co-regulators (Lin *et al*, 2002). Furthermore, transcriptional complexes are not recruited if the AR is bound to an antagonist, such as bicalutamide, thereby preventing phosphorylation and ubiquitination of the AR and so inhibiting gene transcription (Kang *et al*, 2002). Recent studies have also suggested a role for sumoylation in regulating AR activity since it has been shown that SUMO-1 and 3 can both regulate AR activity in prostate cancer cell lines but as yet this is not fully understood (Zheng *et al*, 2006).

1.5 Role for androgens in male reproductive development

1.5.1 Normal androgen action and AR expression

1.5.1.1 Differential effects of testosterone and DHT in the fetal reproductive tract

In some cells, testosterone can be converted to the more potent androgen, DHT by the enzyme 5 α -reductase (see section 1.4.1). Two isoforms of 5 α -reductase exist in mammals, type 1 and 2, which are coded for by 2 genes located on different chromosomes (Andersson and Russell, 1990; Labrie *et al*, 1992). Type 1 is expressed in the skin and liver whereas type 2 is expressed in the fetal and adult male reproductive tract and is the isoform believed to be responsible for male pseudohermaphroditism (Andersson *et al*, 1991; Thigpen *et al*, 1993; Imperato-McGinley and Zhu, 2002). 5 α -reductase mRNA expression is regulated by testosterone (Andersson *et al*, 1989). This can be seen from studies in castrated rats in which the reduced 5 α -reductase expression can be recovered by exposure to exogenous testosterone (Andersson *et al*, 1989). More recently, it has been suggested that this androgen-mediated regulation of 5 α -reductase activity may be due to paracrine/autocrine induction of IGF-1 production in the skin (Horton *et al*, 1993).

Testosterone and DHT regulate the differentiation of different structures during male fetal development: testosterone is known to regulate WD development whereas differentiation of

the UGS and external genitalia is controlled by DHT (summarised in Fig. 1.8) (Wilson *et al*, 1981). However, it is important to highlight that testosterone can mediate differentiation of the external genitalia if it is present in sufficient concentrations, thus suggesting that the differential effects of testosterone and DHT on different structures in the male reproductive tract are due to concentration gradients rather than due to different mechanisms of action. Considerable variation is seen in the ability of different tissues to convert testosterone to DHT (Wilson and Lasnitzki, 1971). For example, the male UGS and urogenital tubercle are able to convert testosterone to DHT before they undergo sexual differentiation to the prostate, external genitalia and urethra, whereas 5 α -reductase activity is not detected in the WD until after sexual differentiation has begun, thus suggesting its early differentiation depends on testosterone rather than DHT (Wilson and Lasnitzki, 1971; Siiteri and Wilson, 1974; Thigpen *et al*, 1993; Tsuji *et al*, 1994). It is interesting to note that the e17 rat female urogenital sinus also expresses 5 α reductase (Berman and Russell, 1993). It is important to note that 5 α -reductase is expressed in the WD later in development, during its morphological differentiation, and is abundantly expressed in the adult epididymis, thus it cannot be stated that DHT has no role in WD development or adult function (Siiteri and Wilson, 1974; reviewed in Robaire and Henderson, 2006). The production of DHT in WD derived structures is especially pertinent in the caudal tips which differentiate into the seminal vesicles and much higher levels of 5 α -reductase activity are notable in this region. For example, by post-natal day (pnd) 2 in mice, 5 α -reductase activity is much lower in the prospective epididymis than in the seminal vesicles or UGS (Tsuji *et al*, 1994; Berman *et al*, 1995). Locally high levels of testosterone are achieved during WD development as it diffuses from the nearby testis and this may compensate for its lower potency compared with DHT. Conversely, structures more distal to the testis depend on circulating testosterone exposure thus amplification of the testosterone signal to the more potent androgen, DHT is required. It is interesting to note that 5 α -reductase activity is much higher in the mesenchymal compartment than in the epithelium in both the UGS and seminal vesicles in pnd0 mice, further highlighting the dependence of androgen action on stromal-epithelial interactions (Tsuji *et al*, 1994; Berman *et al*, 1995). Further investigation has revealed that 5 α -reductase type II, but not type I, is expressed in the developing UGT mesenchyme (Tsuji *et al*, 1994; Berman *et al*, 1995).

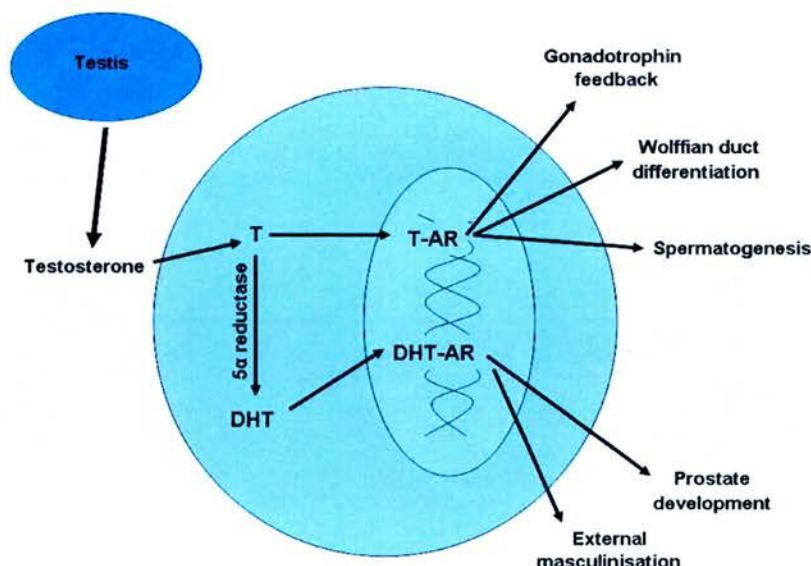


Figure 1.8 Summary of androgen action in fetal male reproductive development (Adapted from Brinkmann, 2003).

Further evidence for the differing roles of T and DHT in different structures in the male reproductive tract has been gained from patients with a deficiency in the 5α -reductase enzyme. Evidence for this disorder was first highlighted in 1974 (Imperato-McGinley *et al*, 1974) and is now known to be due to a mutation in the gene coding for 5α -reductase type II (Andersson *et al*, 1991). This causes pseudohermaphroditism by preventing normal masculinisation of the prostate and external genitalia, but has no obvious impact on the differentiation of the WD into its adult derivative structures (Imperato-McGinley *et al*, 1974; Imperato-McGinley, 2002).

Furthermore, studies undertaken in animal models exposed to the 5α -reductase type II inhibitor, finasteride (Russell and Wilson, 1994) have confirmed the role for DHT in development of the prostate and external genitalia but have shown it is not essential for WD differentiation (George, 1997; Bowman *et al*, 2003). It is worth noting that inhibition of 5α -reductase does not cause such severe prostate involution as does complete androgen ablation by castration and that if testosterone levels are increased sufficiently, DHT is not required for prostate development (Lamb *et al*, 1992). Together these results suggest that testosterone can maintain prostate development, and conversion to DHT is merely a means of locally amplifying the androgen signal from testosterone. High concentrations of testosterone can interact with the AR in a similar manner to DHT and this allows testosterone to be as effective as DHT in stimulating AR activity in spite of having a reduced affinity for binding to the AR (Grino *et al*, 1990).

1.5.1.2 AR expression in the fetal male reproductive tract

It has been suggested that the pattern of androgen receptor (AR) expression reflects the androgen responsiveness of the tissue (Bentvelsen *et al*, 1995). Several studies have mapped AR expression in the fetal reproductive tract highlighting, differences in AR expression in the fetal reproductive tract compared to that observed postnatally (Cooke *et al*, 1991; Bentvelsen *et al*, 1995; Majdic *et al*, 1995; Sajjad *et al*, 2004). ARs are initially present in both the male and female UGS and tubercle, but as they sexually differentiate AR expression increases in males and decreases in females (Bentvelsen *et al*, 1994). Early in male reproductive differentiation, ARs are initially expressed in the mesenchyme of the male reproductive tract with expression not appearing in the epithelium until later, possibly even after the initiation of differentiation. This differs from AR expression seen at later stages of development. This led to the hypothesis that androgens induce epithelial differentiation in the male reproductive tract by acting on the surrounding mesenchyme (Cunha, 1976) (discussed further in section 1.7).

AR protein has been shown to be expressed in the rat male reproductive tract from as early as e14 (Bentvelsen *et al*, 1995), however exact timings of expression in each tissue varies between different studies (Bentvelsen *et al*, 1995; Majdic *et al*, 1995). These differences may be due to the use of different antibodies and immunohistochemical methods and/or different strains of rat. The first tissue to express AR in the rat reproductive tract is the efferent ducts, which are AR positive from e14 (Bentvelsen *et al*, 1995) while the fetal rat testis does not express AR until e17.5 in the interstitial cells and from e18.5 in the peritubular myoid cells; Sertoli and Leydig cells show no significant nuclear staining for AR during fetal life (Majdic *et al*, 1995). AR protein is expressed in the rat WD mesenchyme from at least e16.5 (Majdic *et al*, 1995); it is interesting to note that AR was more densely expressed in the WD mesenchyme on the side furthest from the MD (Bentvelsen *et al*, 1995). Controversy exists over the onset of AR expression in the WD epithelium: Majdic and colleagues (Majdic *et al*, 1995) detected AR in the epithelium from e18.5 in the rat while Bentvelsen and colleagues also detected AR in the epithelium at the epididymal head on e18 in the rat but did not detect AR in the epididymal tail or vas deferens until pnd2 (Bentvelsen *et al*, 1995). These differences may be due to suboptimal immunohistochemistry techniques used by Bentvelsen and colleagues (1995). Furthermore, studies in mice using ³H-dihydrotestosterone steroid autoradiography reported stromal expression from e13 but epithelial expression was not detected until e19 (Cooke *et al*, 1991), much later than found by Majdic and colleagues in rats (Majdic *et al*, 1995). This difference in timing of expression may be due to species differences or different methodologies. The mesenchyme of the

prospective male external genitalia in the rat expresses AR from e14 while the prostate mesenchyme expresses AR from e16: as in other structures in the urogenital system, AR was not detected in the prostatic epithelium until later, at pnd 2 in the rat (Bentvelsen *et al*, 1995). The male and female MD remains immunonegative for AR whereas the regressing female WD is immunopositive for AR at e18 but to a lesser degree than in the male rat (Bentvelsen *et al*, 1995).

1.5.2 Impaired androgen action

Further insight into the role for androgens and the mechanisms involved in male reproductive development can be gained from studying AR mutations and exposure to anti-androgens.

1.5.2.1 Genetic alterations affecting androgen action

1.5.2.1.1 AR mutations

Mutations in the AR result in abnormal sexual development in males. There have been over 300 mutations reported in the human AR (see the international database at <http://www.mcgill.ca/androgenbd>): these mutations are often found at the C-terminus and impair ligand binding but can be observed anywhere in the AR sequence. The first AR mutation to be molecularly characterised was that identified in testicular feminisation (Tfm) mice. Tfm was identified as an X-linked recessive disorder which resulted in genetic males which had testes and normal testosterone production but whose external genitalia were phenotypically female (Lyon and Hawkes, 1970). Further examination established that the MDs regressed normally but that the WD, UGS and external genitalia failed to masculinise in Tfm mice. Furthermore, although both DHT and testosterone are present, cytosolic AR protein could not be detected. This provided the insight that the Tfm mutation caused a complete insensitivity to androgens due to a defect in AR protein, rather than a lack of androgen production (Attardi and Ono, 1974; Gehring and Tomkins, 1974). The Tfm mice have a single base deletion in exon 1 at the amino terminus of the AR leading to a frameshift, introduction of a stop codon and premature termination of AR translation leading to formation of a truncated AR protein downstream of codon 412 (He *et al*, 1991). In vitro translation studies have shown that the Tfm AR cannot produce full length AR protein and it lacks the ability for transcriptional activation since it cannot bind to DNA (He *et al*, 1991). A rat model of Tfm also exists in which there is a mutation in the ligand-binding domain, however controversy exists over the degree of inactivation of the AR (Yarbrough *et al*, 1990).

A wide spectrum of AR mutations has been identified in humans all of which result in some end-organ androgen resistance, a condition known as androgen insensitivity syndrome (AIS). The first evidence for this came from studies showing decreased androgen binding to cultured genital skin fibroblasts of affected individuals (Keenan *et al*, 1974; Wilson *et al*, 1974). It was later established that this was due to mutations in the AR (Hiort *et al*, 1993; Hiort *et al*, 1996). AIS can manifest as complete (CAIS) or partial (PAIS) absence of response to androgens in XY males even though formation of a normal testis has occurred (Quigley *et al*, 1995; Ahmed *et al*, 2000). These patients have normal, or even elevated testicular testosterone production, often associated with increased LH levels (Imperato-McGinley *et al*, 1982; Hughes and Patterson, 1994). The high testosterone levels can be aromatised into oestradiol, which may account for the further lack of masculinisation seen in XY CAIS patients (Brinkmann, 2001). Many mutations have been reported in the AR and can result in a complete absence of any functional AR protein or the presence of qualitatively abnormal AR protein. The clinical observations of AIS vary considerably depending on the nature of, and location of, the mutation (Saunders *et al*, 1992; Hughes and Patterson, 1994; Patterson *et al*, 1994). Four different types of mutation have been identified in the AR in AIS patients: single point mutations in the AR coding sequence resulting in altered amino acid sequences in the ligand-binding or DNA-binding domains, nucleotide insertions or deletions, complete or partial deletion of the gene and intronic mutations affecting AR RNA (Lubahn *et al*, 1989; Ris-Stalpers *et al*, 1990; Marcelli *et al*, 1991; Gottlieb *et al*, 1999). The latter two types of mutation are much less common. It has recently been suggested that the severity of the insensitivity phenotype in individuals is highly correlated to the ability of the AR to shuttle into the nucleus (Wang *et al*, 2005).

Due to the X chromosome recessive trait of AIS, only genetic males are affected while females are carriers of the disorder. Wide variation is reported in the phenotype of affected individuals, with the main focus on the masculinisation of the external rather than the internal genitalia. Patients with complete AIS (CAIS) fail to respond to androgens at all, therefore the genetic male has testes but presents with female genitalia, a blind ending vagina, an absence of WD-derived structures or prostate and gynaecomastia (Hughes, 1997). PAIS patients have partial impairment of AR action, which varies considerably between patients, thus explaining the wide variation in phenotypes observed ranging from phenotypic female genitalia to normal male genitalia but with cryptorchidism and/or oligospermia (Patterson *et al*, 1994). In PAIS individuals, WD-derived tissues can range from partially to fully differentiated depending on the mutation in the AR (Brinkmann, 2001).

It was assumed that if the external genitalia are not masculinised, the WD might not differentiate into the epididymis, vas deferens or seminal vesicles (Hannema *et al*, 2004). However, it has been noted by several researchers that rudimentary internal genitalia persist in some AIS individuals (Morris, 1953; Bale *et al*, 1992; Boehmer *et al*, 2001) suggesting that the WD did not completely regress. Furthermore, Hannema and colleagues (2004) have shown the persistence of well developed epididymides and vas deferens in some CAIS patients. All of these CAIS patients had single amino acid substitutions in the ligand binding domain of the AR: ARs from many of these patients were capable of residual transcriptional activity *in vitro*, unlike ARs with mutations in the DNA binding domain which completely lacked transcriptional activity (Hannema *et al*, 2004). These data suggest that when mutations in the LBD result in protein capable of mediating some degree of androgen responsiveness, the availability of locally high concentrations of testosterone in the WD is capable of rescuing it and even stimulating some degree of differentiation. It is interesting to note that the external genitalia were feminised in CAIS individuals with persistent WDs (Hannema *et al*, 2004), which would be consistent with the action of high local levels of testosterone secreted into the WD from the adjacent testis, while circulating levels may not be sufficient to stimulate external genitalia masculinisation. This is consistent with early experiments undertaken by Jost and colleagues showing that if androgen insufficiency is induced in fetal rabbits prior to sexual differentiation, a gradient of undermasculinisation is observed with structures more distal to the testis being more affected (Jost *et al*, 1953). It is worth pointing out that the levels of testosterone available during sexual differentiation show great variation between individuals which may go some way toward explaining the variation in phenotype of AIS patients (Reyes *et al*, 1973). Furthermore, no information is available regarding the ability to convert testosterone to DHT in the AIS patients with persistent WDs but feminised external genitalia (Hannema *et al*, 2004). Since 5 α reductase enzyme is induced by testosterone, it is possible that this enzyme is not activated in these patients thus explaining the lack of masculinisation of DHT-dependent structures. In addition, Hannema and colleagues (2004) suggest that specific co-factors may be expressed in the WD, which are not expressed in the external genitalia, and these co-factors could mediate and enhance androgen action.

Further insight into the role played by androgens in male sexual development can be gained from sex reversed mice heterozygous for Tfm (Drews and Dieterich, 1978). Sex reversed mice are XX males due to a translocation of the sex determining region of the Y chromosome onto one of their X chromosomes (Cattanach *et al*, 1971). About a quarter of the XX offspring born will be X^{Tfm}/X⁺(Sxr/+); these mice will have testes, due to the Sry

mutation, and male sex organs, due to testosterone production by their testes (Drews, 1975; Drews and Dieterich, 1978). Due to random X inactivation, sex reversed Tfm mice are mosaics with a mixture of androgen insensitive Tfm cells and androgen sensitive wild type cells. This can be seen in the WDs, where the Tfm cells cannot respond to testosterone but they survive as undifferentiated cells due to signalling from the androgen sensitive cells. It is interesting to note that the epididymis of these offspring differentiate and become convoluted (Drews and Alonso-Lozano, 1974; Drews and Dieterich, 1978). However, there are many fewer Tfm cells than wild type cells in the mosaic epididymis, suggesting that many of the Tfm cells undergo apoptosis (Drews and Dieterich, 1978). Further investigation highlighted that, upon testosterone withdrawal by castration, both the Tfm and wild type cells die, indicating that the trophic effect of testosterone arises in the connective tissue of the epididymis rather than the cells themselves (Drews and Dieterich, 1978). These animals have feminised external genitalia and vaginas (Drews, 1975).

1.5.2.1.2 Defects in androgen production

It is not only the presence of androgens that is important in male sexual differentiation but also the level of androgens available at the correct time in development. Any alterations in the genes that code for LH or for any of the enzymes involved in steroidogenesis can result in partial to complete sex reversal. For example, defects in the LH receptor can result in Leydig cell hypoplasia and micropenis (Themmen and Huhtaniemi, 2000) while mutations in 17 β HSD results in complete sex reversal at birth (Lindqvist *et al*, 2001).

1.5.2.2 Effects of gestational anti-androgen exposure on the male reproductive tract

Antiandrogens can act in a variety of ways such as by reducing testosterone production (e.g. phthalates) or by antagonising androgen action by binding to the AR (e.g. flutamide). There are many examples of antiandrogenic compounds, but the effects of just a few commonly studied compounds will be discussed below. Maternal exposure to antiandrogenic compounds during gestation results in varying degrees of impaired masculinisation of the male offspring, some of which can be observed in the fetus and others are not evident until puberty/adulthood.

1.5.2.2.1 Flutamide

Flutamide is a non-steroidal competitive AR antagonist which blocks the action of both testosterone and DHT as flutamide interferes with interactions between the N and C terminals of the AR thus preventing transcriptional activation of AR in target tissues: flutamide has no direct effect on testosterone or DHT production (Peets *et al*, 1974; Schauffele *et al*, 2005).

Flutamide was developed by the pharmaceutical industry as a treatment for androgen sensitive prostate cancer, therefore it was specifically designed as an AR antagonist and its mode of action is well characterised unlike other anti-androgens (McLeod *et al*, 1993). Flutamide is rapidly absorbed after oral exposure and is quickly converted in the liver to the biologically active metabolite 2-hydroxyflutamide (Katchen and Buxbaum, 1975; Zuo *et al*, 2002). It is worth noting that flutamide is quickly cleared from the body and excreted in urine, and has a plasma half-life of just 5-6 h in man (Neri and Monahan, 1972). In male rats that were orally administered flutamide (5 mgkg⁻¹), highest levels of flutamide were noted in tissues just 6 h after exposure and had significantly reduced by 18 h after dosing; neither flutamide nor its metabolites were seen to accumulate in any tissue except the prostate (www.drugs.com). Even though flutamide is believed to be a relatively strong AR competitor, exposure to high doses of flutamide is required to ensure sufficient competition for circulating androgens: studies have shown that flutamide levels 500-1000 fold higher than circulating testosterone levels are required for sufficient antagonism (Simard *et al*, 1986).

As well as its role in treating prostate cancer, flutamide also provides a useful tool to study androgen action in the male reproductive tract. Treatment of pregnant rats with various doses of flutamide (6-300 mgkg⁻¹) between e12-21, showed that there is a dose dependent effect on masculinisation of the male fetuses resulting in varying degrees of WD differentiation, maldescent of the testis, 'feminisation' of external genitalia and missing prostates in adulthood (Imperato-McGinley *et al*, 1992; McIntyre *et al*, 2001). Exposure to flutamide did not result in any significant toxicity to the dam and did not alter the sex ratio (McIntyre *et al*, 2001). It is interesting to note that the prevalence of WD-derived abnormalities varies between different studies. For example, incomplete epididymides were only evident at doses of flutamide above 50 mgkg⁻¹ in a study undertaken by McIntyre and colleagues (2001) whereas earlier investigations by Imperato-McGinley and colleagues (1992) noted this in males exposed to more than 100 mgkg⁻¹; most adult males who had been exposed to 300 mgkg⁻¹ *in utero* had no epididymal body or vas deferens evident (Imperato-McGinley *et al*, 1992). The differences reported in the dose of flutamide required to induce abnormalities in each of the groups is likely to be due to different routes of administration with exposure via gavage resulting in more severe effects than exposure by subcutaneous injection. (Imperato-McGinley *et al*, 1992; McIntyre *et al*, 2001). This is probably due to flutamide being quickly metabolised to the more potent AR antagonist 4-hydroxy flutamide if exposed orally (Xu and Li, 1998). *In utero* exposure to just 12.5 mgkg⁻¹ flutamide resulted in complete absence of the prostate in adulthood (McIntyre *et al*, 2001), suggesting that DHT-dependent

structures are more sensitive to the effects of flutamide exposure than are testosterone-dependent structures. This is possibly due to the higher concentrations of androgens available locally from the testis to compete with flutamide in the developing WD compared to the UGS (Veyssiere *et al*, 1982). To the best of my knowledge, no information is available regarding flutamide's ability to preferentially compete with testosterone or DHT to bind to the AR. The animal studies described above suggest two possibilities. Firstly, flutamide competes more easily with DHT for AR binding therefore impairing DHT-dependent development more readily than testosterone-dependent development. This appears illogical, however, since DHT has a stronger affinity for the AR than does testosterone. Secondly, flutamide competes equally with testosterone and DHT for binding to the AR, but as there are more testosterone molecules than DHT present in the WD to compete with flutamide, some testosterone is still able to bind to the AR. Since DHT is more potent, fewer molecules are needed in DHT-dependent structures and so there are fewer molecules present to compete with flutamide, i.e. it is simply about abundance of androgen molecules to compete with flutamide for AR binding. Further investigation is required to distinguish these possibilities. Furthermore, it is not known whether flutamide exposure prevents testosterone-dependent activation of 5 α reductase, therefore further impairing development of DHT-dependent tissues.

1.5.2.2.2 Linuron

Linuron is a urea-based herbicide which competes with endogenous androgens for binding to the AR: this binding, however, is with lower affinity than that seen with other AR antagonists such as flutamide or vinclozolin, thus making it a weaker antagonist (Waller *et al*, 1996; Lambright *et al*, 2000). In vitro, linuron competitively antagonises AR transcriptional activity induced by DHT (McIntyre *et al*, 2000). Experiments in rats to examine the impact of maternal exposure to linuron (50-100 mgkg⁻¹) during the window of fetal reproductive development suggest that linuron antagonises androgen-mediated reproductive development since anogenital distance (AGD) was reduced, nipples persisted in males, and 7% of adult male offspring had incomplete epididymides (Gray *et al*, 1999; McIntyre *et al*, 2000). It is worth noting, however, that linuron exposure preferentially impaired testosterone-mediated reproductive development rather than DHT-mediated events (McIntyre *et al*, 2000). This is in contrast to the impact of other AR antagonists such as flutamide which affects all androgen-dependent development, but with DHT-responsive tissue being more easily affected than testosterone-responsive structures (Imperato-McGinley *et al*, 1992). Some researchers have suggested that these differences are due to

differences in metabolism since flutamide is metabolised to the more potent AR antagonist 4-hydroxyflutamide which persists in the body (Xu and Li, 1998) whereas linuron is slowly metabolised to weaker AR antagonists (Cook *et al*, 1993). Initial analysis of fetal males from dams exposed to linuron (50 mgkg⁻¹) during gestation showed that fetal serum and intratesticular testosterone levels were not affected by linuron exposure and these males had grossly normal WDs, morphologically indistinguishable from controls (McIntyre *et al*, 2002); however, subsequent studies by another group showed a decrease in fetal rat testicular testosterone production after linuron exposure (Wilson *et al*, 2004). WD abnormalities were not seen until pnd7, whereas AGD was reduced in males at e19 and e21 (McIntyre *et al*, 2002). It is not immediately clear why fetal linuron exposure does not alter epididymal development until early in postnatal life. The normal rat epididymis continues to coil and differentiate postnatally, therefore it would seem that antagonism of androgen action during fetal life can have an impact on subsequent postnatal development, even though exposure has ceased at birth. Additional studies by the same group reported that in the majority of e21 males there were no obvious WD abnormalities but reduced coiling was seen in around a quarter of linuron-exposed males and 20% had an incomplete WD (Turner *et al*, 2003). It is puzzling why these abnormalities were not detected fetally until the latter study, but the investigators claim this to be due to improved morphological analysis techniques and variability between litters (Turner *et al*, 2003). Taken together, however, these results suggest linuron-induced abnormalities are atypical of an AR antagonist and more closely resemble phthalate-induced effects. Why this should be the case is not readily apparent. Effects of linuron exposure on the male fetal reproductive tract were not as severe as those seen after exposure to other antiandrogens and vary considerably between different studies therefore it could be suggested that linuron is not a particularly useful tool for studying androgen receptor antagonism and the resulting effects on the fetal WD. Turner and colleagues (2003) went on to examine any changes in gene expression in WDs at e21 from linuron-exposed male fetuses and showed that AR, Notch 2, IGF-1R and EGFR mRNA were reduced, while Dlk (a potential ligand for Notch2) was increased. These changes may be indicative of disruption of the paracrine interactions between stromal and epithelial cells which subsequently results in impaired WD development.

1.5.2.2.3 Vinclozolin

Vinclozolin is a fungicide commonly used in the fruit industry but has been shown to have antiandrogenic properties (Kelce *et al*, 1994). Vinclozolin is capable of binding to the AR thus competing with androgens and inhibiting androgen-dependent gene expression (Kelce *et*

al, 1997). Maternal exposure to vinclozolin (200 mgkg⁻¹) inhibits masculinisation of the male fetuses in rats, resulting in reduced AGD, hypospadias, the presence of a blind-ending vagina and small or no accessory sex glands (Gray *et al*, 1994). Wolf and colleagues exposed pregnant rats to vinclozolin during various 2 day windows during the period of fetal reproductive development and noted that the most sensitive period during fetal life for exposure to vinclozolin (400 mgkg⁻¹) was between e16-17: extended exposure between e14-19 resulted in more severe, and an increased prevalence of, abnormalities (Wolf *et al*, 2000). Exposure to vinclozolin for just 2 days between e12-13, 14-15, 16-17, 18-19 or 20-21 did not result in any obvious abnormalities in WD-derived structures in adulthood (Wolf *et al*, 2000). This is not surprising since Gray and colleagues (1994) saw no epididymal defects in rats exposed to vinclozolin (200 mgkg⁻¹) between e14-pnd3.

1.5.2.2.4 Di(n-butyl) phthalate

DBP is a phthalate ester which is widely used in the plastics and other industries: exposure to DBP impacts on male reproductive health, with neonates more sensitive to the effects than adults (Foster *et al*, 1980). Unlike many other anti-androgens, such as flutamide and vinclozolin, phthalates do not appear to act directly via the AR but cause their effects by reducing fetal testicular testosterone production (Mylchreest and Foster, 2000; Parks *et al*, 2000; Fisher *et al*, 2003). Various studies have shown that maternal exposure to 100 mgkg⁻¹ DBP or above during fetal life (e12-21) in rats results in undermasculinisation of the fetal male offspring in a dose dependent manner (Mylchreest *et al*, 1998; Mylchreest *et al*, 1999; Mylchreest *et al*, 2000; Foster *et al*, 2001). The most marked effects reported are on epididymal development, but other adverse effects include cryptorchidism, hypospadias, reduced AGD, malformed external genitalia and abnormal or underdeveloped prostate, seminal vesicles and other secondary sexual organs (Mylchreest *et al*, 1998; Mylchreest *et al*, 1999; Mylchreest *et al*, 2000; Foster *et al*, 2001). An interesting point to note is that phthalate exposure interferes with testis descent by inducing regression of the gubernaculum. Testis descent is believed to be a bi-phasic event which depends on the action of both testosterone and the peptide hormone insulin-like peptide (insl3) and phthalates are the only antiandrogen so far found to be capable of reducing both testosterone and Insl3 levels (Wilson *et al*, 2004).

1.5.3 The effects of exogenous androgen exposure on fetal reproductive development

As discussed above, impaired androgen action in fetal life interferes with normal male reproductive development. However, it is widely accepted that excess androgen exposure

during fetal life also perturbs normal fetal development, resulting in masculinisation of the female reproductive tract (reviewed in Abbott *et al*, 2005). This view has arisen from both studies in animal models and clinical observations.

1.5.3.1 Clinical observations

The most common cause of female masculinisation in humans is congenital adrenal hyperplasia (CAH), which results in most cases from an autosomal recessive mutation that impairs the 21-hydroxylation reaction. This causes a deficiency in cortisol synthesis in the fetal adrenal gland such that there is a compensatory increase in adrenal androgen synthesis. This in turn masculinises the fetal female genitalia in XX individuals resulting in an enlarged clitoris, partly fused labia majora, and a common urogenital sinus instead of a separate urethra and vagina. The internal female organs are usually normal and WD-derived structures are not present (reviewed in Merke and Bornstein, 2005). As discussed above, masculinisation of the WD in animals requires high levels of local androgen action. In contrast, the female CAH fetus is exposed to circulating androgens synthesised in the adrenal, rather than androgens delivered locally from the testis, as occurs in fetal males: this may explain the masculinisation of the external but not the internal genitalia in CAH females. To the best of my knowledge, no information is available regarding the ability of these individuals to convert testosterone to DHT but it is assumed that 5 α reductase activity is evident in these patients. XY males can also be affected by CAH and although they have no obvious signs of CAH at birth, they undergo early virilisation at 2-4 years old (reviewed in Merke and Bornstein, 2005).

It is hypothesised from both animal studies and clinical observations, that polycystic ovary syndrome (PCOS) could be due to excess androgen exposure during fetal life. Symptoms of PCOS can be seen in adult female rhesus monkeys and sheep that have been exposed *in utero* to levels of testosterone equivalent to fetal male levels (Abbott, 1998; Padmanabhan, 1998). These symptoms include hypersecretion of luteinising hormone, abnormal insulin secretion, enlarged cystic ovaries and anovulation. However, PCOS patients do not present with masculinised external genitalia suggesting they were exposed to lower levels of testosterone that were insufficient to masculinise the reproductive tract but which were sufficient to affect ovarian development.

1.5.3.2 Animal studies

Several studies have been undertaken in animals to examine the effects of exogenous androgen exposure during fetal life. Exposing pregnant rats to various doses of testosterone masculinises the female offspring to varying degrees (Swanson and Werff ten Bosch, 1965;

Ogawa and Nozawa, 1969; Jost *et al*, 1973; Stinnakre, 1975; Wolf *et al*, 2002; Wolf *et al*, 2004). It has been shown that early in gestation, male and female fetuses have similar plasma androgen levels but as gestation proceeds, androgen levels significantly decrease in females and rise in males to peak at around e18-19 in rats (Weisz and Ward, 1980; Baum *et al*, 1991). At this age, plasma androgen levels are approximately 60% higher in males than in females (Baum *et al*, 1991). It is interesting to note that these studies report a reduction in plasma androgen levels in male fetuses at e20 resulting in similar plasma androgen levels in both male and female rat fetuses at e20 and 21 (Weisz and Ward, 1980; Baum *et al*, 1991). The authors claim that this short peak in androgens in males is sufficient to sensitise the male fetus to subsequent androgen exposure and initiates male sexual differentiation. These authors claim this plasma androgen comes mainly from the placenta in the female fetuses, as the rat placenta has been shown to be capable of testosterone synthesis from progesterone or pregnenolone in the second half of gestation (Chan and Leathem, 1975; Gibori and Sridaran, 1981). It has been suggested that this peak in androgens sensitises the developing male in order that it can respond to androgens during neonatal and postnatal life (Baum *et al*, 1991). It is somewhat surprising that females are exposed to low levels of androgens early in gestation since they also express ARs thus are capable of responding to androgens (Bentvelsen *et al*, 1995).

The effects of exogenous androgen exposure on different reproductive structures depends on the timing and dose of testosterone given. Furthermore, injection of testosterone into the pregnant dam has less effect on the developing female than does injection directly into the amniotic sac or into the fetus itself (Swanson and Werff ten Bosch, 1965). Care should be taken when comparing the levels of testosterone used in these earlier studies as they are often expressed as testosterone dose per rat rather than by dam weight or as the total testosterone exposure over the entire dosing period. Prenatal exogenous androgen exposure has no obvious effect on the male reproductive tract but masculinises the female reproductive tract, with AGD and the external genitalia most sensitive to these effects (Greene, 1939; Swanson and Werff ten Bosch, 1965; Ogawa and Nozawa, 1969; Wolf *et al*, 2002). It is interesting to note that exposure to low levels of testosterone can masculinise the external genitalia and stimulate development of the prostate and seminal vesicles in females but does not compromise normal reproductive function (Greene, 1939; Ogawa and Nozawa, 1969). Rescue and differentiation of the WD in females requires higher levels of testosterone exposure than does masculinisation of the UGS or external genitalia and exposure must be prior to e18 in the rat (Swanson and Werff ten Bosch, 1965; Ogawa and Nozawa, 1969; Stinnakre, 1975). As in CAH patients, this is likely to be due to the testosterone being

delivered from the circulation rather than locally as occurs naturally in the WD in males, therefore testosterone levels do not reach sufficient levels to masculinise the WD. Many previous studies do not comment on the phenotype of the WD but merely its presence or absence. However, Greene and colleagues (1939) noted that exposure to high doses of testosterone (80-90 mg total dose) was able to not only rescue the WD but could even stimulate some degree of coiling in the epididymal segment, while lower doses (13.5 – 40 mg total dose) could often only rescue the WD unilaterally with the adult derived structures often incomplete and lacking patent lumens. Unfortunately, images were not provided to allow visualisation of this coiling. As with other studies, the UGS was well masculinised in these low dose testosterone-treated animals, again highlighting the increased sensitivity of these structures to androgen action (Greene, 1939).

A recent study by Wolf and colleagues (2002) showed that exposure to testosterone propionate (TP) below 0.5 mg/pregnant rat had little effect on the developing reproductive tract but exposure to 0.5 – 10 mg/pregnant rat resulted in a permanent increase in female AGD, absence of a vaginal orifice, masculinised external genitalia, and the presence of a prostate and seminal vesicles. It is worth noting that they were unable to rescue the WD in these female offspring, even with 10 mg/rat TP. These organ-specific differences in masculinisation via TP exposure are probably due to the circulating testosterone being converted to DHT in the more distal structures such as the prostate and external genitalia whereas the WD does not express 5 α reductase so depends on high levels of local testosterone to stimulate differentiation. Follow up studies by the same group showed that combined exposure to testosterone (1 mg/rat) and vinclozolin (200 mgkg⁻¹) resulted in normal female reproductive development suggesting that this dose of vinclozolin is sufficient to antagonise the effects of testosterone, but the dose of testosterone was unable to prevent the effects of vinclozolin on the male fetuses (Wolf *et al*, 2004). It can be seen from these studies therefore that testosterone alone is sufficient to rescue the female WD, but there is considerable variation in the doses and timing of exposure reported to elicit this masculinisation. Furthermore, little information is available detailing the phenotype of the rescued WD.

It is worth mentioning that it is not only the reproductive tract which can be masculinised in female fetuses exposed to exogenous androgens, it can also impact on brain development resulting in an increase in male-like play behaviour in rats, for example, increased rough play was noted in juvenile females that had been exposed to testosterone *in utero* (Meaney and Stewart, 1981). Testosterone exposure also induces intrauterine growth retardation (IUGR) (Wolf *et al*, 2002; Wolf *et al*, 2004; Steckler *et al*, 2005; Carlsen *et al*, 2006;

Padmanabhan *et al*, 2006). It is known that inappropriate steroid hormone signalling during fetal life can affect neonatal growth and examples of this can be seen in humans, as patients with polycystic ovarian syndrome (PCOS) have an increased prevalence of babies born small for gestational age (Sir-Petermann, 2005). It is unknown, however whether this is the result of testosterone action directly in the mother or is the result of testosterone being converted into oestradiol, since oestrogens are also known to cause a decrease in maternal weight gain and fetal birth weight, at least in rats (Zimmerman *et al*, 1991; Padmanabhan *et al*, 2006).

Further evidence for extraneous testosterone exposure masculinising female fetuses comes from studies in mice in which the Wnt-4 gene has been deleted (Vainio *et al*, 1999). In contrast to Wnt-4 mutant males, which have normal reproductive tracts, Wnt-4 mutant females do not develop normally but are masculinised (Vainio *et al*, 1999). It has been shown that their ovaries synthesise testosterone which, in turn, rescues the WD and even stimulates coiling in the proximal segment: contrastingly, the external genitalia of these mice are not masculinised (Vainio *et al*, 1999). The difference in the structures masculinised in these mice compared to animals exposed to maternal exogenous testosterone is likely due to the differences in the source of testosterone i.e. local or circulating. Furthermore, in female Wnt4 mutant mice, the levels of circulating testosterone are lower than in normal males thus are presumably insufficient for masculinising structures distal to the source of testosterone i.e. the ovary. It is not immediately obvious why this would be the case but perhaps the levels of circulating testosterone are insufficient to induce 5 α reductase and so DHT activity. The Müllerian duct cannot be identified in males or female Wnt4^{-/-} animals at birth: taken together with the evidence demonstrating that the Müllerian ducts form from Wnt4 expressing coeleomic epithelium and that the mesenchyme underlying the Müllerian duct expresses Wnt4, this suggests that Wnt4 is necessary for Müllerian duct formation (Vainio *et al*, 1999).

1.6 Role for oestrogens in urogenital development

Oestrogen synthesis is temporarily activated in the male and female embryo prior to implantation in the uterine wall, suggesting oestrogen action is vital for embryo survival (George and Wilson, 1978). Oestrogen synthesis is later re-initiated in the fetal ovary prior to histological differentiation suggesting that female reproductive differentiation depends at least partly on oestrogen action (Wilson *et al*, 1981). Oestrogen action is mainly mediated by binding to intracellular receptors, either oestrogen receptor α (ER α) or β (ER β) (Evans, 1988). These receptors are members of the steroid nuclear receptor family (discussed in section 1.5) and act as ligand dependent transcription factors (reviewed in Evans, 1988; Couse and Korach, 1999). ER β protein is highly homologous to ER α protein, particularly in

the DNA-binding domain (Kuiper *et al*, 1996). The biological significance of the two ER subtypes is still unclear, but it is likely that they may play different roles in different tissues, possibly responding to different ligands since they only share around 60% homology in the ligand-binding domain (Kuiper *et al*, 1996).

Many studies have been undertaken to examine the expression of ER α and ER β , however, this review will focus only on their expression in the rodent reproductive tract. Most studies in the reproductive tract have investigated adult ER expression showing that both receptors are expressed in most structures in the male. These studies highlighted that ER β is more abundantly expressed and in more cell types than is ER α in the rat male reproductive tract (Saunders *et al*, 1998). For example, little ER α is expressed in the adult rat prostate while ER β protein was localised to the secretory cells: similarly, ER α was weakly expressed in adult rat seminal vesicles whereas ER β was detected in both epithelial and stromal cells (Pelletier *et al*, 2000). ER α is expressed in the epididymal epithelium and in the stroma of the vas deferens at pnd18 but this expression is lost by adulthood: contrastingly, ER β is expressed in the epithelium and stroma of the rat epididymis and vas deferens throughout postnatal life (Atanassova *et al*, 2001). Neonatal rat exposure to DES, a synthetic oestrogen, alters this ER expression pattern which may account for the 'blurring' of the boundary between the vas deferens and epididymis seen in these animals (Atanassova *et al*, 2001).

Both male and female fetuses express ER α and β , with all cells that express AR in the developing rat reproductive tract also expressing one or both of the ERs (Williams *et al*, 2001): ER β is believed to be more highly expressed in the developing male reproductive tracts than is ER α (Sharpe, 1998). In the mouse, ERs are present in the WD mesenchyme, but not the epithelium, from e16 and expression decreases as these mesenchymal cells differentiate into smooth muscle (Stumpf *et al*, 1980; Cooke *et al*, 1991). The efferent ducts are the first site of ER expression in the epithelium (by e16) with epididymal expression beginning later at e19: the vas deferens epithelium remains negative for ERs (Cooke *et al*, 1991). However, these studies did not differentiate between ER α and β as they were prior to the discovery of ER β and used steroid autoradiography.

As discussed above, it is widely accepted that fetal WD development is dependent on androgens but there is some evidence for a role for oestrogens in some aspects of reproductive tract development, especially around the time of puberty. For example, neonatal exposure to exogenous oestrogenic compounds results in abnormal development or even atrophy of the epididymis (Orgebin-Crist *et al*, 1983; Robaire, 1988; Williams *et al*, 2001). However, although neonatal exposure to DES results in structural abnormalities in the testis and reproductive tract prepubertally, these can largely be prevented by co-

administration of testosterone (Williams *et al*, 2001). It was hypothesised therefore that the adverse changes induced by DES exposure are the result of a disturbed androgen-oestrogen balance, and that rather than the absolute hormone levels being the determining factor, it is the relative levels of androgens and oestrogens that is important in determining whether such abnormalities develop (Williams *et al*, 2001). It is worth noting that DES-induced abnormalities are always associated with reduced AR expression, therefore highlighting the possibility that DES is acting effectively as an anti-androgen (Williams *et al*, 2001; Williams *et al*, 2001).

Further insight into the role for oestrogens in the male reproductive tract has been gained from studies in mice lacking ER β (β ERKO) or α (α ERKO) (Lubahn *et al*, 1993; Krege *et al*, 1998). Oestrogens are believed to play a role in the adult male reproductive tract (Hess *et al*, 1997; Hess *et al*, 2001), however, as yet there is no direct evidence for a role in the development of this tract. Male α ERKO mice are infertile whereas male β ERKO are fertile (Lubahn *et al*, 1993; Krege *et al*, 1998). Epididymides from young α ERKO mice have abnormal cell organisation and impaired fluid reabsorption by the efferent ducts, which may explain the abnormal sperm maturation observed in these mice (Eddy *et al*, 1996; Hess, 2000; Hess *et al*, 2000). It is unlikely, however, that oestrogens play a role in WD rescue or morphological differentiation since both α ERKO and aromatase knockout (ArKO) mice have normal looking epididymides present at birth (Fisher *et al*, 1998; Robertson *et al*, 1999; Hess *et al*, 2000). A role for oestrogens in WD functional differentiation cannot be completely ruled out, however, since adult efferent ducts and epididymides do not appear to function normally in α ERKO mice (Hess *et al*, 2000).

1.7 Stromal-epithelial interactions

Stromal-epithelial interactions are believed to be vital in mediating the steroid-dependent regulation of reproductive tract development. In both fetal and adult life, the organs of the male and female reproductive tract are composed of epithelial parenchyma and surrounding supporting fibromuscular stromal or mesenchymal cells (Fig. 1.9). Communication between cells is vital in multicellular organisms to coordinate differentiation, growth, motility and immunity and interactions between epithelial and mesenchymal cells are known to play vital roles in embryonic development and in adulthood in both normal and abnormal tissues (e.g. cancer) (Cunha, 1976; Cunha *et al*, 1985).

For the purpose of this review, the term “stroma” will refer to both the fetal mesenchymal cells as well as the postnatal stromal cells.

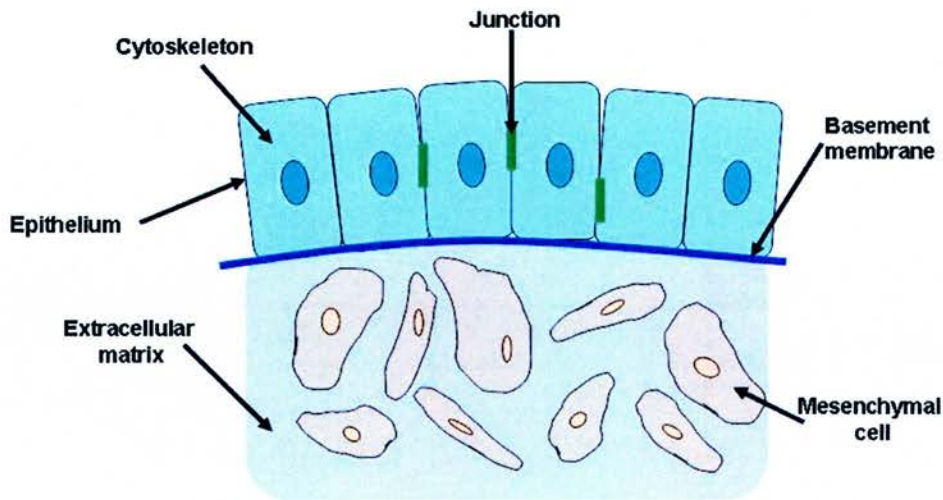


Figure 1.9 Schematic representation of the spatial organisation of stromal and epithelial cells in reproductive structures.

1.7.1 Epithelium

The epithelium is the basic tissue of early embryos in which cells are tightly bound together with specialised junctions between them connecting them into a sheet of epithelium: these sheets line all the cavities and free surfaces of the body and serve several fundamental purposes including acting as a barrier to the external environment, regulating pattern formation and providing mechanical integrity (summarised in Alberts, 1994). Epithelial cells have little ECM between them but rest upon an underlying basement membrane: this is a specialised layer of ECM which provides the interface between the stromal and epithelial compartments and plays a role in maintaining tissue integrity (discussed in section 1.8). Furthermore, cytoskeletal filaments cross the cytoplasm between cells, and along with junctions, give the epithelium strength and structure: these filaments are discussed further in section 1.8.

1.7.2 Stroma

Mesenchymal cells, the embryonic equivalent of stromal cells, firstly arise in the embryo by migrating from the primitive streak (reviewed in Hay, 1995; Shook and Keller, 2003). Definitive embryonic mesenchyme often arises by epithelial-mesenchymal transformation (EMT) from the mesodermal epithelium (reviewed in Hay, 1995; Shook and Keller, 2003). This cell type transformation can occur in either direction and plays a considerable role in normal developmental processes that require migration and ECM invasion as well as in disease development such as cancer (reviewed in Shook and Keller, 2003; Singh *et al*, 2003). For example, epithelial cells can turn off epithelial markers such as cytokeratin, activate

stromal master genes and gain motility thus allowing them to interact with the ECM and gain stromal-like properties. Alternatively, stromal cells can de-differentiate to re-express epithelial regulatory genes such as E-cadherin, turn off motility machinery and regain polarity (reviewed in Hay, 1995; Thiery, 2002; Kang and Massague, 2004).

The stromal compartment is thought of as a loose fibrous connective tissue which is made up of a variety of different cell types including fibroblasts, endothelial cells, smooth muscle cells and neuroendocrine cells. These cells are surrounded by a structural ECM, which plays a vital role in its function. The ECM and its role in stromal-epithelial interactions is further discussed in section 1.8.

1.7.3 Stromal-epithelial interactions in the reproductive tract

The development of the fetal reproductive tract depends on reciprocal interactions between the epithelial and stromal cells and if either component is removed or altered, tissue morphogenesis is disrupted (reviewed in Franks *et al*, 1970; Marker *et al*, 2003; reviewed in Cunha *et al*, 2004). Classical experiments carried out by Cunha and colleagues have highlighted the direct role for the stroma in inducing epithelial differentiation in the male and female reproductive tract (reviewed in Cunha *et al*, 1985; reviewed in Cunha *et al*, 2004). In the fetus, paracrine signals from stromal cells specify the identity of, and induce differentiation of, epithelial cells as well as dictating the morphological pattern of the epithelium, thus controlling whether the epithelium forms a highly branched network such as in the prostate or a simple unbranched tube such as in the WD (Donjacour and Cunha, 1991; Cunha, 1998). The stroma is also able to regulate the timing, pattern and rate of epithelial proliferation and apoptosis as well as the functional differentiation of the epithelium in the developing reproductive tract (Cunha and Young, 1992; Kurita *et al*, 2001; Marker *et al*, 2003). Much of this evidence comes from studies undertaken by Cunha and colleagues which examined the effects of recombining stroma from one reproductive organ with epithelium from another, highlighting that the fetal stroma can dictate the phenotypic differentiation of the epithelium (Cunha *et al*, 1985; Cunha and Young, 1991; Cunha *et al*, 1992; Cunha and Young, 1992; Cunha *et al*, 1992; Cunha *et al*, 1997; Cunha *et al*, 2004). For example, epithelia from the upper and middle portions of the WD develop into seminal vesicles, rather than epididymides and vasa deferentia, when recombined with neonatal seminal vesicle stroma (Higgins *et al*, 1989). This mesenchyme was able not only to regulate cytodifferentiation of the epithelium but even induced the secretion of seminal vesicle secretory proteins showing induction of functional differentiation (Higgins *et al*, 1989).

These observations raise the question of whether this fetal specification and differentiation of the epithelium is permanent or whether adult epithelium remains susceptible to re-specification by changes in the signals from the mesenchyme. In adult organs, the epithelium is generally thought of as stably differentiated but these cells are continually cycling and being replaced, therefore epithelial morphology and function must be maintained and cell proliferation tightly regulated in order to sustain organ integrity. It is likely that this occurs by similar mechanisms to those operating in the fetus involving stromal-epithelial interactions. Previous studies have shown that adult epithelia remain responsive to signalling from the surrounding stroma. For example, fetal UGS stroma can stimulate adult prostate epithelium to produce new ducts (Chung and Cunha, 1983; Cunha *et al*, 1983). Similarly, adult mammary epithelium initiates morphogenesis in response to fetal mammary stroma (Sakakura *et al*, 1979).

Earlier studies showed that adult epithelia are not only responsive to signalling from the stroma but that they are not irreversibly committed to their phenotype and can be re-specified to another phenotype by the underlying stroma. For example, adult vaginal epithelium can be differentiated into prostatic epithelium by fetal UGS stroma (Cunha *et al*, 1985). However, it is not just fetal stroma that can alter epithelial morphology but adult stroma can also elicit major changes in the epithelium. This has been shown in experiments using adult vagina stromal cells which can induce both the morphological and functional re-differentiation of neonatal uterine epithelium into vaginal epithelium (Cunha, 1976). These results are not surprising since adult epithelium continues to be renewed and replaced throughout life, therefore it needs to retain its plasticity and ability to respond to its environment.

It has therefore been well documented that epithelial differentiation, growth and morphogenesis, in both adults and fetuses, depends upon interactions with the underlying stroma. However, studies highlight that the interactions between these two cell types are reciprocal and that the epithelium can signal to the stroma and facilitate its capacity to respond to subsequent hormonal stimulation. For example, in males prostatic epithelial differentiation depends on androgen action on the stroma but stromal differentiation into smooth muscle depends on reciprocal androgen-dependent signalling from the epithelium (Cunha *et al*, 1996). Further evidence for this phenomenon comes from recombination studies whereby uterine, but not vaginal, epithelium is able to promote uterine stromal proliferation in response to oestrogen and progesterone (Bigsby, 2002), and if uterine epithelium is ablated *in vivo*, the stroma is no longer capable of decidualising in response to hormones (Lejeune *et al*, 1981).

1.7.4 Role of stromal versus epithelial steroid receptors in stromal-epithelial interactions

The studies discussed above highlighting the vital interaction between stromal and epithelial cells suggest that hormone receptors expressed in either the stromal or epithelial compartment may play a differential role in hormone signalling during reproductive development and/or function. This can be seen during prostate development: initially ARs are only expressed in the developing stroma prior to bud development, thus suggesting that androgens act on the stroma which then signals to the epithelium to regulate its differentiation (Cooke *et al*, 1991). This has been further studied in recombination studies *in vitro* using stroma and epithelium from mice lacking steroid receptors or from wild type mice (Cunha *et al*, 1987; Cooke *et al*, 1997; Buchanan *et al*, 1998; Kurita *et al*, 1998). For example, studies recombining Tfm stroma with wild type epithelium did not result in prostate formation in response to androgens *in vitro* whereas wild type stroma grown alongside Tfm epithelium resulted in prostate development and differentiation (Cunha *et al*, 1987) (summarised in Fig. 1.10). These studies therefore highlight that androgen-responsive prostate development is mediated through ARs in the stroma which can then stimulate the epithelium to proliferate and differentiate, even if the epithelium has no AR.

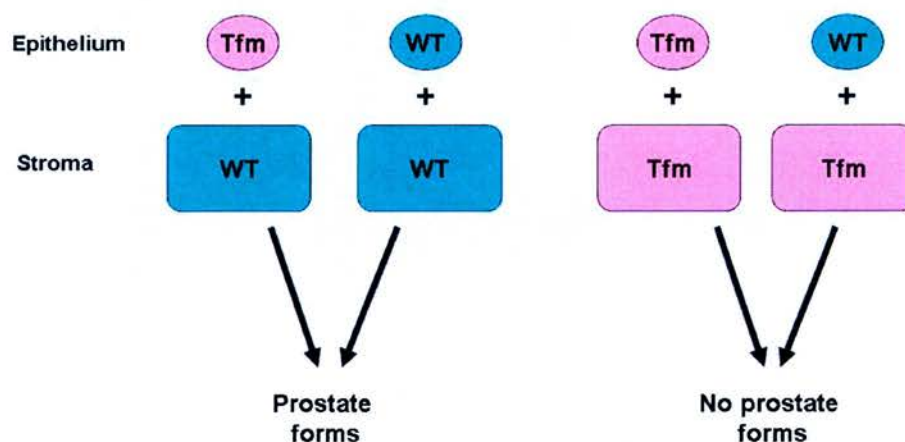


Figure 1.10 Summary of a typical recombination experiment carried out by Cunha *et al*. Wild type (WT, blue) tissue expresses androgen receptor whereas testicular feminised (Tfm, pink) tissue does not.

Furthermore, studies have been carried out to investigate whether androgen-dependent effects on the prostate epithelium continue to be mediated via the stroma in the adult, as occurs in the fetus. Wild-type UGS stroma was combined with wild-type or Tfm fetal bladder epithelium and transplanted under the kidney capsule in adult male host mice (Cunha

and Chung, 1981). Both types of recombination resulted in the growth of mature prostatic buds which degenerated in response to castration (Cunha and Chung, 1981). Subsequent testosterone replacement was able to restore prostate morphology and stimulate epithelial proliferation, thus highlighting that, even in the recombinants in which the epithelium was AR negative, the prostate could respond to androgens and elicit epithelial proliferation (Sugimura *et al*, 1986). As in the fetus, this must be mediated via the ARs in the stroma, further highlighting the vital role for steroid receptors and stromal-epithelial interactions.

However, care must be taken in interpretation of these experiments as they could suggest that direct steroid hormone action on the epithelium is not essential during reproductive development. This does not appear to be true as further investigations have revealed that epithelial steroid receptors are required for functional differentiation. For example, epithelial AR expression is necessary for expression of AR-dependent secretory proteins in seminal vesicle epithelium (Cunha and Chung, 1981) and ER α expression in both the epithelium and stroma is vital for epithelial stratification and cornification in the vagina (Buchanan *et al*, 1998).

1.7.5 Role for stromal-epithelial interaction in disease

Stromal-epithelial interactions have also been highlighted in several disease states including cancer (Cunha *et al*, 2002). In adulthood, homeostasis of organs is believed to be maintained by interactions between the stroma and epithelium to regulate proliferation and apoptosis (Cunha *et al*, 1985; Arnold *et al*, 2001) and disruption of these interactions can result in de-differentiation of prostatic cells, de-regulation of proliferation and apoptosis and thus development of cancer (Cunha *et al*, 1996; Cunha *et al*, 2002). Once again, recombination experiments have highlighted the role for steroid receptors and stromal-epithelial interactions in disease states. In some cases, receptors were required in both the stroma and epithelium to induce metaplasia: for example, in the prostate oestrogens acting through ER α cause squamous metaplasia of the epithelium, however ER α must be expressed in both the epithelium and the stroma for this to occur (Risbridger *et al*, 2001). In this system, it is hypothesised that the stromal ER α mediates proliferation of the epithelium while epithelial ER α regulates epithelial differentiation. This differs from the mechanism seen in the development of cervicovaginal adenosis which depends only on epithelial receptors, DES exposure acting through ER α in the epithelium to induce cervicovaginal adenosis (Kurita *et al*, 2004).

1.7.6 Potential mediators of stromal-epithelial interactions

It is unknown whether stromal-epithelial interactions rely on direct contact between the stroma and epithelium or whether some soluble diffusible factor such as growth factors may be responsible. Early cell culture studies showed that epithelial cells could not survive *in vitro* in the absence of stromal cells, but that extracellular matrices could be used in place of cells to support epithelial cells and promote proliferation (Cunha *et al*, 1985). However, direct contact with stromal cells was required to stimulate epithelial differentiation and morphogenesis suggesting that the ECM plays a role in these stromal-epithelial interactions but some stromal-specific factors are required for more complex epithelial differentiation. These factors are obviously stromal factors and are likely to be tissue specific since different stroma can promote different epithelial morphology and function.

1.7.6.1 Role for growth factors in stromal-epithelial interactions

One suggestion is that growth factors may be involved in regulating stromal-epithelial interactions. Many growth factors have been shown to be present in the male reproductive tract including FGF, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and keratinocyte growth factor (KGF), with several researchers implicating roles for them in both the adult and developing reproductive tract (Bossert *et al*, 1990; Cunha *et al*, 1992; Alarid *et al*, 1994; Cunha *et al*, 1995; Gupta, 1996; Sugimura *et al*, 1996; Chua *et al*, 2002; Wang *et al*, 2003). The general mechanisms behind growth factor signalling extend beyond the scope of this review but have been extensively reviewed recently, however as yet, the exact function, intracellular signalling pathways and mechanisms involved are poorly understood (Tomsig and Turner, 2006; Tomsig *et al*, 2006).

The most extensively studied growth factor in male reproductive development is epidermal growth factor (EGF). Several studies have shown that blocking EGF action results in WD abnormalities and degeneration of the epithelium, even in the presence of endogenous androgens, and that EGF is able to mimic some of the effects of testosterone including partially rescuing the female WD and increasing female AGD (Gupta *et al*, 1991; Cain *et al*, 1994; Gupta, 1996). Furthermore, exposure to EGF can partially rescue the effects of flutamide exposure both *in vivo* and *in vitro* (Gupta *et al*, 1991; Cain *et al*, 1994). It appears that the effects of EGF and androgens are reciprocal in that EGF stimulates androgen binding and modulates AR transcriptional activity in the reproductive tract, possibly by promoting phosphorylation of the p160 family coactivator transcription intermediary factor 2/glucocorticoid receptor interacting protein 1 (TIF2/GRIP1) and increasing its interaction with AR (Gupta *et al*, 1996; Gregory *et al*, 2004): on the other hand, androgens stimulate EGF secretion, induce EGF receptor (EGFR) expression, induce phosphorylation of EGFR,

and establish the ability to secrete and bind EGF in response to androgens (Traish and Wotiz, 1987; Kashimata *et al*, 1988; Gupta and Jaumotte, 1993; Johnson *et al*, 1993). It is interesting to note that EGF cannot induce AR Ser-213 phosphorylation in the absence of an AR agonist (Taneja *et al*, 2005). EGF mRNA levels peak in the male mouse reproductive tract at e14 and e18, coinciding with the onset of WD stabilisation and differentiation, respectively, thus highlighting the potential importance of EGF in WD development (Gupta and Singh, 1996). EGFR protein has been identified in the mouse male reproductive tract from e18 and appears to increase with age and testosterone exposure, whereas flutamide exposure can decrease EGFR expression (Gupta, 1996). The EGFR protein is expressed in the mouse WD epithelium rather than the stroma, while EGF ligand can be detected in both the stroma and the epithelium suggesting a role for both paracrine and autocrine EGF signalling (Winters *et al*, 1993; Gupta, 1997). Together with the fact that early in sexual differentiation ARs are only expressed in the stroma, this suggests that EGF may be involved in mediating the androgen signal from the stroma to the epithelium and enhancing AR transcriptional activity. Complete masculinisation therefore appears to depend on both testosterone and EGF, possibly due to EGF mediating the effects of testosterone (Gupta and Jaumotte, 1993; Gupta, 1996; Gupta *et al*, 1996).

Another growth factor commonly studied in male reproductive development is KGF, which has been shown to be widely produced by the stromal cells and to act specifically on epithelial cells (Alarid *et al*, 1994). Previous studies have indicated a role for KGF in mediating androgen-driven stromal-epithelial interactions in seminal vesicle development (Alarid *et al*, 1994). As with EGF, blocking KGF in organ culture inhibited epithelial proliferation and differentiation but had no obvious impact on the stroma (Alarid *et al*, 1994). Furthermore, KGF is believed to mediate androgen action between the stroma and epithelium in prostate cancer (Planz *et al*, 1999).

Taken together, these studies all point to a role for growth factors in mediating androgen dependent stromal-epithelial interactions during male reproductive development. However, it remains questionable whether growth factors alone can induce the specificity required for tissue morphogenesis as they are often ubiquitously expressed.

1.7.6.2 Role for Hox genes in stromal-epithelial interactions

The factors regulating stromal-epithelial interactions and specifying epithelial identity are unlikely to be transiently expressed since the epithelium appears to remain relatively plastic throughout life and depends on constant signalling from the underlying stroma to maintain its phenotype. Hox genes may fulfil this role as their expression in the reproductive tract is patterned in the fetus and persists into adulthood suggesting Hox genes may play a role in

maintaining tissue patterning postnatally. Hox genes are homeobox containing transcription factors which regulate patterning and segmentation of the body axis in the fetus (Favier and Dolle, 1997). These genes are highly conserved and bind to specific sequences of DNA to modulate gene transcription (Levine and Hoey, 1988). Late in gestation, the 5' members of the Hox gene family are expressed in the male reproductive tract (Dolle *et al*, 1991) and this expression persists in the adult with Hox a10, a11, a13, d10 and d13 expressed in the epithelium and stroma of the postnatal epididymis and vas deferens (Hsieh-Li *et al*, 1995; Podlasek *et al*, 1999; Bomgardner *et al*, 2001; Bomgardner *et al*, 2003). These genes have been suggested to play a role in patterning the reproductive tract as mice deficient in Hox a10 show reduced budding in their seminal vesicles (Podlasek *et al*, 1999) while mice lacking Hox a11 are infertile with homeotic transformation of the vas deferens to more closely resemble an epididymal phenotype (Hsieh-Li *et al*, 1995).

Hox gene expression has been shown to be regulated by sex steroid hormones in the endometrium with Hox A10 and A11 both increasing in response to oestrogen or progesterone (Taylor *et al*, 1998; Taylor *et al*, 1999). Furthermore, exposure to the potent oestrogen, DES, *in utero* interferes with the normal pattern of Hox gene expression in the female reproductive tract, resulting in anterior homeotic transformation (Block *et al*, 2000). For example, Hox a10 is normally expressed in the stroma and epithelium of the whole uterus but exposure to DES *in utero* restricts expression to the caudal segment of the uterus only (Block *et al*, 2000). These results suggest that one mechanism by which DES alters female reproductive patterning is by altering Hox gene expression and thus tissue identity. This raises the possibility that sex steroids may regulate the expression of Hox genes in both the stroma and the epithelium in the male reproductive tract which in turn will mediate tissue patterning and morphogenesis in the fetus. Together these data suggest that Hox genes may play a role in mediating the hormone-dependent stromal-epithelial interactions in the reproductive tract.

1.8 Extracellular matrix and its role in reproductive development

The extracellular matrix (ECM) surrounds cells and is vital not only to provide mechanical support but it also plays a role in controlling their behaviour, proliferation and differentiation by regulating the availability of signals, such as growth factors, reaching the cell surface (Streuli and Gilmore, 1999). The ECM is composed of fibres, including collagen, fibronectin and elastin, and ground substance, which includes glycosaminoglycans such as hyaluronic acid or heparan sulphate. In most connective tissues, the supporting fibroblasts secrete the majority of the components of the ECM however some are made by the epithelium such as hyaluronic acid.

1.8.1 ECM and stromal-epithelial interactions

It has been suggested that the ECM plays a role in mediating the signals between stromal and epithelial cells and thus plays an important role in inducing and regulating organogenesis and tissue remodelling. For example, hyaluronic acid plays a role in organ formation by creating free space for cells to migrate into, thus allowing a structure to change shape (summarised in Alberts, 1994).

Early attempts at cell culture suggested that epithelial cells could not be maintained *in vitro* in the absence of stromal cells as the epithelial cells flattened and de-differentiated when cultured alone on glass or plastic surfaces (Cunha *et al*, 1985). However, if these epithelial cells were cultured on collagen or other biomatrices, they maintained their functional differentiation and morphology in the absence of stromal cells (Lee *et al*, 1984). These and other studies led to the hypothesis that, as well as the stromal effects caused by secreted paracrine factors (as discussed in section 1.7.6), at least some of the effects of stromal cells on the epithelium may be due to, or act via, the ECM. Cunha suggested that more complex morphogenetic processes may require the presence of stromal cells while maintenance and proliferation of differentiated adult epithelial cells may be mediated by the ECM alone (Cunha *et al*, 1985). This can be seen in cultured adult mammary epithelial cells as they require the presence of mammary stromal cells in order to induce ductal morphogenesis *in vitro*: collagen substrates alone are not sufficient to cause morphogenesis but are able to stimulate epithelial proliferation (Sakakura *et al*, 1979; Cunha *et al*, 1985). These data imply that the integrity of the ECM is fundamental in tissue morphogenesis by playing a vital role in signal transduction between stromal and epithelial cell compartments both in the fetus and the adult.

1.8.2 Basement membrane

The basement membrane is a special thin tough sheet of ECM which forms the interface between the epithelium and the surrounding connective tissue or stroma. The basement membrane plays an important role in mediating epithelial cell adhesion, polarity, gene expression, proliferation, migration and differentiation (Danen and Yamada, 2001). Integrity of the basement membrane is vital to maintain tissue structure and loss of the basement membrane can result in epithelial cell death and epithelial-stromal transition, as seen in the regressing Müllerian duct (discussed in section 1.3.1.1) (Dyche, 1979; Ikawa *et al*, 1984; Inomata *et al*, 1989; Austin, 1995).

The basement membrane is made up of 3 layers: the lamina lucida which lies nearest the tissue it is supporting (i.e. the epithelium), the middle dark lamina densa, and the lamina

fibroreticularis which merges with the supporting tissue (i.e. the stroma) (summarised in Alberts, 1994). The basement membrane is mainly made by the cells that rest upon it (i.e. epithelia) and is composed normally of type IV collagen, heparan sulphate and structural glycoproteins (summarised in Alberts, 1994). These structural glycoproteins include fibronectin, entactin and laminin and bind the basement membrane and the underlying connective tissue to the cell membrane it is supporting (Erickson and Couchman, 2000). Laminin is one of the first ECM proteins made in the developing embryo with early embryonic basement membranes mainly composed of laminin with little or no collagen IV (Dziadek and Timpl, 1985). Laminin is widely expressed, produced mainly by epithelial cells and is involved in a variety of processes such as adhesion, migration, embryo development and wound healing (Aumailley and Smyth, 1998).

Fibronectin is another extracellular adhesion protein however it promotes attachment of fibroblasts to the ECM, rather than epithelial cell attachment as does laminin. Fibronectin is secreted by fibroblasts and accumulates along the interface between the epithelium and stroma and attaches cells to a variety of extracellular matrices, except type IV matrices that involve laminin as the adhesive molecule (Ruoslahti, 1981). Fibronectin is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion. Fibronectin sometimes serves as a general cell adhesion molecule by anchoring cells to proteoglycan substrates or organises cellular interactions with the ECM by binding to different components of the extracellular matrix and to membrane-bound fibronectin receptors on cell surfaces (summarised in Alberts, 1994). Like many other cytoskeletal proteins, fibronectin plays a role in regulating cell structure and behaviour and redistribution of fibronectin can be associated with epithelial differentiation (Paranko *et al*, 1983). The importance of fibronectin in cell migration events during embryogenesis has been documented in several contexts: for example, during vertebrate limb development the pattern of fibronectin deposition dictates the patterns of precartilaginous cell adhesion to the ECM, thus determining limb-specific patterns of chondrogenesis (Tavella *et al*, 1997). Changes in fibronectin levels have also been associated with altered epithelial cell proliferation in response to hormones in the mammary gland (Woodward *et al*, 2001). Furthermore, fibronectin has been reported to increase matrix-metalloproteinase 2 (MMP-2) activity and induce epithelial cell loss in mammary gland involution after hormone withdrawal (Schedin *et al*, 2000). Taken together, these results suggest that the basement membrane and its components are vital in maintaining tissue integrity and interruption to the basement membrane can result in loss of the epithelium and therefore degeneration of the tissue.

1.9 Cytoskeleton

As well as the ECM regulating cell behaviour and hence organogenesis, all mammalian cells have a cytoskeletal network in their cytoplasm which also plays a vital role in maintaining cell shape, allowing cell movement and division and mediating intracellular transport (summarised in Alberts, 1994). This cytoskeleton is composed of microtubules, actin filaments and intermediate filaments (IF) (summarised in Alberts, 1994). There are 5 types of IF: Type I & II – Cytokeratins, which are found in epithelial cells; Type III – Vimentin, desmin, peripherin, which provide mechanical strength to cells; Type IV – neurofilaments, which strengthen the long axons of neurons; and Type V – lamins, which stabilise the inner membrane of the nuclear envelope (summarised in Alberts, 1994).

Despite their chemical diversity, IFs all play similar roles providing a supporting framework within the cell, connecting the membrane and nucleus and thus regulating protein localisation and transmission of signals (Paramio and Jorcano, 2002). IFs could therefore play a vital role in organogenesis, more specifically in mediating signals between the stroma and epithelium, and disruption to the cytoskeleton could result in impaired inter-cellular signalling and thus lead to degradation of the tissue. IFs interact with proteins which can regulate their behaviour thus causing IF re-organisation or collapse: these proteins are often signal transducers and their interaction with IFs can alter the signalling potential and hence alter tissue morphogenesis (Paramio and Jorcano, 2002).

Expression of the early stromal IF, vimentin, is often transient with its expression preceding expression of other type III IFs during differentiation: for example, vimentin is expressed in all fibroblasts and is believed to play a role in cell motility, but it is often subsequently replaced by desmin as the cells differentiate into muscle cells. However, aberrant expression of vimentin can result in abnormal cell behaviour. This can be seen in epithelial cells that have metastasised in which vimentin is re-expressed and cytokeratin expression is lost, thus explaining the increased invasive capacity of the cells (Singh *et al*, 2003). This increase in vimentin and decrease in cytokeratin in epithelial cells is a sign of epithelial-stromal transformation, as discussed above. During this process, a decrease in cell adhesion molecules is also often observed, especially E-cadherin (Avizienyte *et al*, 2002).

1.10 Conclusions

It is widely accepted that androgens play a role in mediating WD stabilisation and development, however the mechanisms, the precise timing of events and the downstream signalling pathways involved in this remain somewhat of a mystery. The experimental work undertaken in this thesis focuses on the upper segments of the WD and the mechanisms involved in both its initial rescue in males and its subsequent differentiation into the epididymis and vas deferens. These adult derivative organs are vital for the maturation and transport of sperm, therefore normal development of these structures is fundamental for normal male fertility. It has been shown by many researchers that impaired androgen action in the fetus interferes with this developmental process. However, most researchers have examined the effects in adult tissues, rather than in the fetus, and as yet the mechanisms involved are not understood. It is feasible that stromal-epithelial interactions, possibly acting via the ECM, are likely to be involved since they play a major role in mediating the development and differentiation of other tissues in both the male and female reproductive tract.

Over 20 years ago, Wilson and colleagues (1981) wondered how tissues develop the capacity early in fetal life to respond to hormonal stimulation later in development and how the same hormonal signal can be translated into different physiological effects in different tissues. This phenomenon remains unanswered today but insight into this will help us to understand the mechanisms involved in male reproductive development and more specifically, WD development.

This previous research led us to hypothesise that androgens control WD stabilisation and differentiation by acting on the stroma and mediating the patterning and coiling of the future epididymis via stromal-epithelial interactions. The primary aim of this thesis was therefore to investigate the impact on the fetal WD of impaired androgen action. Pregnant rats were exposed to the anti-androgen flutamide and the WDs from the male offspring were examined at both the gross and histological level (Chapter 3). Chapter 4 focuses on the further characterisation of the histological changes in the fetal WD after flutamide exposure using markers of normal WD development and compartmentalisation. Previous studies have interfered with androgen action during the entire window of male reproductive development (e14-21), therefore studies were designed to investigate the precise window of androgen action in WD development by examining the effect of flutamide exposure either during the initial window of stabilisation (e14-17) or during morphological differentiation of the WD (e19-21) in the rat (Chapter 5). Since the WD naturally regresses in female fetuses, this

provided a unique opportunity to examine the impact on the WD in the male of artificially reducing androgen action compared to a naturally occurring model (Chapter 6). These studies were performed in parallel with an investigation of impaired WD development in animal models with more complete reduction in androgen action, namely Tfm mice in which the AR is inactive and rats exposed to DBP + flutamide in which both testosterone synthesis and action are impaired (Chapter 6). The natural model of female WD regression was further utilised to examine whether exogenous testosterone exposure was sufficient to rescue and differentiate the female WD or whether some other testicular factor also plays a role in male WD development (Chapter 7). Finally, chapter 8 summarises and discusses the main findings of this research and examines the contribution made to our further understanding of the mechanisms and timing of WD development.

1.11 Aims

The main aims of this thesis are:

To examine the role for androgen action in fetal WD development.

To investigate the cellular and molecular mechanisms involved in fetal WD development and their perturbation by anti-androgen treatment.

To investigate the role for stromal-epithelial interactions in WD development.



2 General Materials and Methods

The aim of this project was to investigate the role for androgens in the development of the Wolffian duct (WD). This involved the use of control rats for investigating the timing of natural WD development and regression as well as for *in utero* experiments manipulating androgen action. Details on the sourcing of the biological tissues, their handling and their experimental processing are provided in this chapter. Where specific procedures were used, further information is provided in the relevant experimental chapter.

2.1 Animal work

All animal studies were carried out in accordance with the Animal (Scientific Procedures) Act 1986. Wistar rats were bred and maintained in our own animal facility, the BRF, provided by the University of Edinburgh. Daily animal husbandry and the majority of the licensed procedures were carried out by Mark Fiskien of the MRC, for which I am extremely grateful. All procedures were undertaken according to UK Home Office regulations, under project license 60-3045, held by Prof Richard Sharpe.

2.1.1 Welfare conditions

Animals were housed under standard conditions and had access *ad libitum* to fresh tap water and a soy-free breeding diet (Type 3, soy free, rat; SDS diets; Dundee, UK). Light was provided from 7am until 7pm daily, humidity was kept at 55% and the temperature maintained between 20–25°C.

Rats were housed in clear cages with solid bottoms, contained bedding, consisting of wood shavings and corn cob, and a cardboard tunnel for environmental enrichment (BS&S, Scotland). Up to six females were housed together after mating whereas stud male rats were singly housed, except during mating.

For experiments that allowed dams to deliver pups and pups to develop postnatally, dams were individually housed just prior to parturition until weaning of the pups after 21 days old.

2.1.2 Time-matings

Grid bottomed cages were used for mating to allow copulatory plugs to fall through the cage and so be detected on trays below. Time-matings were set up to allow accurate calculation of their stage of gestation. One stud male and one female rat were paired together in grid bottom cages at 08.00 and monitored hourly for the presence of a vaginal plug. Detection of a copulatory plug was taken as evidence of mating: this was defined as embryonic day 0.5

(e0.5). Once a positive mating was detected, the female was removed from the male rat. If mating was not detected by 16.00, males were removed from the mating cage overnight and pairings set up again at 08.30 the following day. Stud males were derived from the in-house colony and were between 6-12 months of age at the time of mating. Stud males were retired from the colony as plugging efficiency decreased, approximately every 3-6 months. Dams were at least 10 weeks old at mating and where possible, proven dams were used. This standardisation of husbandry practices helped to maximise the reproducibility of the experimental work.

2.2 In utero treatments

Pregnant dams were randomly allocated to treatment groups, summarised in Figure 2.1. Dams were dosed once daily between 08.30-10.00 by either gavage or subcutaneous injection and control dams were treated daily with the vehicle alone. Dams were weighed just prior to treatment to enable dosing of accurate bodyweight related volumes. Dams were checked daily for signs of toxicity and any treatment related changes in animal welfare. Control litters were dosed alongside each new series of treatments. Throughout this thesis, offspring from dams exposed to any of the following compounds during pregnancy will be referred to a treated “in utero” or “flutamide/testosterone/DBP exposed”. This does not mean that the animals were directly exposed but that fetuses were exposed indirectly via the pregnant dam.

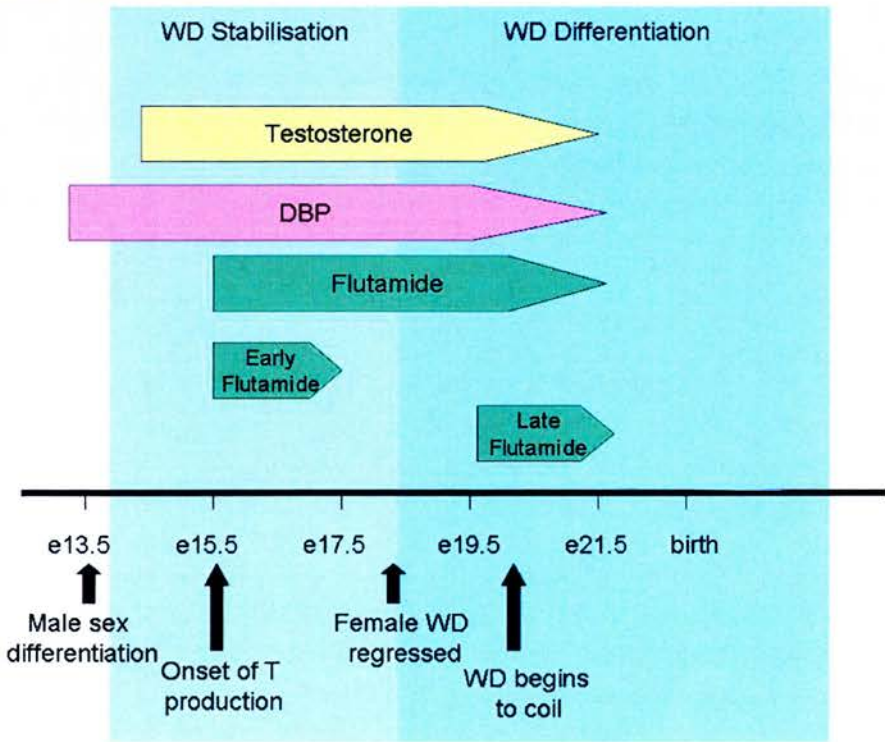


Figure 2.1 Summary of the maternal treatment regimes used.

2.2.1 Gavage

Dosing by gavage is a Home Office recognised route and caused minimal discomfort to the rats. The appropriate volume of test compound was taken up in a disposable plastic 1ml syringe (B-D Plastipak) and transferred directly into the pregnant adult female rats' stomach using the attached 10-12cm long 15-16G commercial blunt ended steel gavage cannula (Medicut, Sherwood Medical Industries Ltd., UK).

2.2.1.1 Flutamide

Flutamide (Sigma, Poole, UK), a competitive androgen receptor antagonist, is a yellow powder stored at room temperature. Flutamide is insoluble in oil therefore was dissolved in DMSO (2.5%; Sigma) prior to diluting in pure corn oil (supermarket bought) to 1 mlkg⁻¹ maternal bodyweight. For example, to dose at 100mgkg⁻¹ bodyweight, 100mg flutamide was dissolved in 25 µl DMSO then made up to 1 ml with corn oil, and administered at a volume of 1mlkg⁻¹ maternal weight. Flutamide was freshly prepared for each experiment and stored at room temperature between dosing: any excess was disposed of appropriately at the end of each study.

Flutamide was administered by gavage to pregnant rats on e15.5-e21.5 at 50 mgkg⁻¹ (n=24 dams) or 100 mgkg⁻¹ (n=23) bodyweight. These doses were selected based on results previously reported highlighting a high incidence of male reproductive tract malformations without any adverse toxicological effects (Imperato-McGinley *et al*, 1992). Dosing was undertaken from e15.5 as this is when the fetal testis begins androgen synthesis (Warren *et al*, 1972).

2.2.1.2 DBP

Di-*n*-butyl phthalate (DBP), CAS No.: 84-74-2, a viscous pale yellow oil, stored at room temperature (Sigma D-2270, >99% pure). DBP is lipid soluble therefore was diluted in pure corn oil to 1 mlkg⁻¹ dam bodyweight. For example: to dose at 500 mlkg⁻¹ maternal bodyweight, 5 ml of DBP was made up to 10ml with corn oil, and administered at a volume of 1 mlkg⁻¹ maternal bodyweight. DBP solutions were freshly prepared for each experiment and were stored at room temperature between administrations: any excess was disposed of appropriately at the end of each study.

DBP was administered by gavage to pregnant rats from e13.5-e21.5 at 500 mgkg⁻¹ bodyweight (n=22). Previous studies have undertaken dose response studies exposing pregnant rats to 4, 20, 100 or 500 mgkg⁻¹ DBP and have shown that exposure to 500 mgkg⁻¹ did not result in any adverse toxicological effects in the dam or increased fetal mortality but

did cause a high incidence of male reproductive tract abnormalities (Ema *et al*, 1997; Mylchreest *et al*, 2000; Foster *et al*, 2001).

2.2.2 Subcutaneous injection

Dosing by subcutaneous injection is a Home Office recognised route and caused minimal discomfort to the rats. The appropriate volume of test compound was taken up in a disposable plastic 1ml syringe (B-D Plastipak) and injected into the pregnant adult female rat using a 25G needle.

2.2.2.1 Testosterone Propionate

Testosterone propionate (TP; T-1875; Sigma) is a solid white powder that is insoluble in water and is stored at room temperature. TP was dissolved in pure corn oil (supermarket bought) to 5 or 20 mlkg⁻¹. For example, to dose at 20 mgkg⁻¹ maternal bodyweight, 20 mg TP was dissolved in 0.4 ml corn oil, and administered at a volume of 0.4 mlkg⁻¹ maternal bodyweight. TP was freshly prepared for each experiment and stored on a Cozee Comfort heating mat (Burco Dean Appliances Ltd, Burnley, UK) set at setting 1 (29°C) between dosing to prevent precipitation of the TP out of solution. Any excess TP was disposed of appropriately at the end of each study.

TP was administered by subcutaneous injection to pregnant rats on e14.5-e21.5 at 5 mgkg⁻¹ (n=8 dams) or 20 mgkg⁻¹ (n=18) maternal bodyweight. These doses were selected based on results previously reported showing that doses above 1 mg TP (per rat) increased female fetal T by 80%, increased female ano-genital distance (AGD) and rescued the seminal vesicles without a high incidence of toxicological effects (Wolf *et al*, 2002).

2.3 Necropsy

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation and fetuses recovered at e17.5 – e21.5. Neonates were killed by decapitation and postnatal animals, older than pnd10, were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Fetal (e21.5), neonates and postnatal animals were weighed and AGD was measured using digital callipers (Faithfull Tools, Kent, UK) (detailed in section 2.3.3).

2.3.1 Gross dissection

2.3.1.1 Fetuses

Fetuses were removed from the dam using an established gross dissection technique. The pregnant dam was positioned supine and the abdomen opened to allow removal of the intact

uterus. Fetuses were removed from their amniotic sac and their umbilical cord was cut prior to decapitation and immersion of the body in ice-cold phosphate buffered saline (PBS, Sigma). Fetuses were transported in PBS on ice to minimise degeneration prior to fine dissection and tissue recovery.

2.3.1.2 Postnatal rats

The abdomen of the supine postnatal male rat was opened and testes and epididymides pulled out of the scrotal sac by the fat pad. The vasa deferentia were cut midway down and the testes, epididymides and vasa deferentia removed from the rat. Males were examined for the presence and morphology of the seminal vesicles and prostate.

2.3.2 Fine dissection

2.3.2.1 Fetal dissections

Gonads and reproductive tracts were recovered from fetuses by microdissection and examined using a Leica MZ6 dissecting microscope. Gonads were trimmed off the WD or Müllerian duct (MD) and reproductive tracts were photographed using a Leica ICA camera. Reproductive tracts from control and treated males and females were analysed microscopically, at the time of dissection, for any gross morphological abnormalities. Considerable variation was seen in the degree of regression of the WDs, therefore careful note was taken of any macroscopic abnormalities, including the degree of coiling and interruption of the WD lumen. This was particularly relevant in fetuses at e21.5 when greater damage and variation was seen than in younger fetuses.

2.3.2.2 Postnatal dissections

Gonads were carefully dissected off the reproductive tracts and epididymides and vasa deferentia were analysed for any gross morphological abnormalities. Reproductive tracts were photographed using a D70 camera (Nikon, Surrey, UK) fitted with a Nikon AF Nikkor 24-120mm lens (Nikon).

2.3.3 Measurement of anogenital distance

The distance between the anus and the base of the genital tubercle (anogenital distance, AGD) increases in males in response to androgens and it is widely believed that AGD reflects the degree of androgenisation/masculinisation of the animal (reviewed in Swan *et al*, 2005; reviewed in Edwards *et al*, 2006). Prior to recovery of reproductive tracts, AGD was measured in fetal (e21.5) and postnatal animals using digital callipers (Faithfull Tools, Kent, UK). This involved measuring the distance between the base of the genital tubercle and the

anus. These measurements were undertaken separately by two independent scientists and average values were recorded.

2.4 Tissue preservation

Isolated fetal tissue was preserved by snap-freezing or fixed in Bouin's or 4% paraformaldehyde (PFA) while postnatal epididymides and vasa deferentia were fixed in Bouin's. All fixatives were prepared by the core histology facility, for which I am very grateful (section 2.14).

2.4.1 Frozen tissue

Isolated male WDs were snap frozen in 1.5 ml cryovials on dry ice then archived at -80° until required.

2.4.2 Tissue fixation

2.4.2.1 PFA for whole mounts

Isolated male and female fetal reproductive tracts were fixed in 4% PFA in an airtight container for 24 h at 4° C prior to dehydration in 25% methanol in PBS, 50% methanol in PBS, 75% methanol in PBS then 100% methanol. Fixed reproductive tracts were stored in methanol in air tight containers at -20°C.

2.4.2.2 Fixation in Bouin's

Isolated male and female fetal reproductive tracts were fixed in Bouin's for 1 h at room temperature in an air-tight container while postnatal epididymides and vasa deferentia were fixed in Bouin's for 6 h. Bouin's fixed tissue was subsequently transferred into 70% ethanol and processed for 17.5 h in an automated Leica TP1050 processor (Leica Microsystems, Milton Keynes, UK). Processed tissue was embedded by hand in liquid paraffin wax and the cooled wax block was stored at room temperature until required. Fetal and postnatal reproductive tracts were deliberately orientated in the paraffin wax to lie horizontally thus standardising the plane of section obtained and allowing complete cross-sections of the whole epididymis to be obtained. This was carried out in our core histology laboratory by Mike Millar, Sheila MacPherson and Arantza Esnal, for which I am extremely grateful.

2.5 Histological analysis

Gross histological analysis was performed on WD sections stained with haematoxylin and eosin, using standard protocols. Note was taken of any histological abnormalities including abundance of epithelial cross-sections, swollen lumens and epithelial malformation. Harris's

haematoxylin (section 2.14) stains cell nuclei blue. Slides were immersed in a bath of haematoxylin for 5 min, rinsed in tap water, briefly immersed in 1% acid alcohol to remove any non-specific cytoplasmic staining and rinsed in tap water. Slides were immersed in Scott's tap water for 30 s to allow the blue dye to develop. This was checked using a standard light microscope. Eosin (section 2.14) was used to stain cell cytoplasm pink. Slides were immersed in a bath of eosin for 30 s then rinsed thoroughly in tap water. The pink colouration was checked using a standard light microscope. Following staining, slides were rinsed in tap water and dehydrated before mounting with a coverslip secured with Pertex™ as described below.

2.6 Protein analysis

2.6.1 Immunohistochemistry

Immunohistochemistry was performed on isolated reproductive tracts, using standard avidin peroxidase protocols, to highlight the location of proteins of interest. All immunostaining, or its absence, was reviewed in the context of the tissue morphology and treatment, alongside proper experimental control tissue. The principles applied and the protocols used are outlined below.

- dewaxing and rehydration of sections
- retrieval of the target antigen for detection by the specific primary antibody
- blocking non-specific antigens
- incubation of tissue with specific primary antibody
- detection of the primary antibody by an amplification system
- visualisation of antibody localisation
- counterstaining of non-immunostained tissue

2.6.1.1 Immunohistochemistry on sections

2.6.1.1.1 Sectioning

Wax blocks containing fixed tissue were chilled on ice prior to sectioning to make them more rigid and thus easier to cut. 5 µm sections were cut using a microtome (Leica, model RM 2135) and floated in a waterbath (Lamb RA, model E/65) at 45-50°C to smooth out any wrinkles. Sections were then mounted onto electrostatically charged glass slides (BDH, Cat No.: 406/0179/00). Slides were stacked in a metal rack and dried overnight in an oven at 50-

60°C (Lamb RA, model E28.5) to ensure sections were adhered to the glass slides. Slides were cooled and stored at room temperature in a dust free environment.

2.6.1.1.2 Dewaxing and rehydrating

Sections were deparaffinised in HistoClear (National diagnostics, Hull, UK) at room temperature for 5 min, x2. Tissue was rehydrated by immersion in 100% ethanol for 30 s x2, 95% for 30 s, 75% for 30 s then washed in water.

2.6.1.1.3 Antigen retrieval

Fixatives induce cross-linking in order to preserve tissue integrity, however, this can often mask proteins (antigens) and thus prevent their immunodetection. In order to unmask these antigens, sections were exposed to high temperature and pH change as detailed by Shi and colleagues (1993). Antigen retrieval was performed using 0.01M citrate buffer, pH 6.0 in a domestic pressure cooker (Tefal, Clypso). Sections were immersed in 2 l of boiling buffer, pressure cooked for 5 min at full pressure, left to stand for 20 min then cooled under running water.

2.6.1.1.4 Blocking

Immunohistochemistry can result in non-specific binding due to non-specific affinity of the primary or secondary antibody or detection reagents to molecules other than the desired antigen. This non-specific binding was blocked deliberately before the primary antibodies were added.

Horseradish peroxidase amplification was used (section 2.6.1.1.6) therefore endogenous peroxidase activity must be blocked to prevent non-specific binding. This was carried out by immersing sections in 3% hydrogen peroxide in methanol (30% H₂O₂, BDH) for 30 min on a rocker at room temperature. Slides were washed in a rocking bath of tap water for 5 min.

Non-specific binding of the secondary antibody was prevented by incubating sections in a dilute solution of serum from the species in which the secondary antibody was raised. In most cases, a goat raised secondary antibody was used therefore slides were incubated in normal goat serum (NGS; Autogen Bioclear UK Ltd, Wiltshire, UK) diluted 1:4 in TBS containing 5% bovine serum albumin (BSA; Sigma). Slides were removed from water and tissue was used to carefully dry around the section to remove excess buffer. Blocking serum was added to sections and slides were maintained in a humidity chamber at room temperature, for 30 min.

Slides were washed well between each step in order to removed residual reagents. All washes between antibody or reagent incubations comprised two 5 min washes on a rocker (20-40 rpm) at room temperature in TBS (section 2.14).

2.6.1.1.5 Primary antibodies

Blocking buffer was replaced on the sections with a solution of primary antibody diluted in blocking serum to an optimised concentration. The slides were incubated overnight in the humidity chamber at 4°C. The exact conditions (concentration/temperature/duration) were optimised for each primary antibody used. Table 2.1 below summarises the general conditions used. Control sections were incubated with blocking peptide when available or blocking serum alone to confirm antibody specificity.

Table 2.1 Summary of primary antibodies used for immunohistochemistry. Santa Cruz products supplied via Autogen Bioclear UK Ltd, Wiltshire, UK.

Target Antigen	Source	Dilution	Retrieval	Host Species
Androgen Receptor	Santa Cruz	1:50	Citrate	Rabbit
Smooth Muscle Actin	Sigma	1:10000	None	Mouse
Pan-cytokeratin	Sigma	1:200	Citrate	Mouse
Phospho-Histone H3	Upstate Biotechnology	1:1000	Citrate	Rabbit
Cleaved Caspase 3	Cell Signalling	1:200	Citrate	Rabbit
Desmin	DAKO	1:400	Citrate	Mouse
Vimentin	DAKO	1:1000	Citrate	Mouse
Laminin	Abcam	1:100	Citrate	Rabbit
E-cadherin	BD Biosceinces	1:200	Citrate	Mouse
N-cadherin	Zymed	1:500	Citrate	Mouse
CD45	BD Bioscience	1:50	Citrate	Mouse
CD68	DAKO	1:500	Citrate	Mouse
Neutrophil Elastase	DAKO	1:500	None	Sheep

2.6.1.1.6 Secondary antibodies

After washing off the primary antibody, sections were incubated with the appropriate secondary antibody to amplify the localisation of the primary antibody. The secondary antibody was raised against a species-specific sequence on the primary antibody. A biotin

labelled goat secondary antibody was diluted 1:500 in blocking serum and incubated on sections for 30 min at room temperature.

Table 2.2 Summary of secondary antibodies used for immunohistochemistry

Target Antigen Species	Host Species	Supplier
Rabbit	Goat	DAKO
Mouse	Goat	Sigma
Goat	Rabbit	Vector

This biotinylated secondary antibody signal was amplified by incubation for 30 min with avidin-biotin conjugated with horseradish peroxidase diluted in 0.05M Tris-HCl, pH 7.4 according to manufacturers instructions (ABC-HRP; DAKO, Ely, UK). This system takes advantage of avidin and biotin having a very high and specific binding affinity for each other (Fig. 2.2).

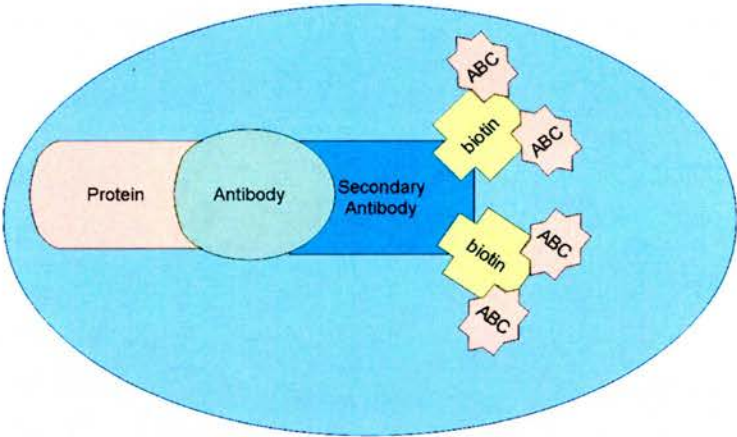


Figure 2.2 Schematic representation of the principles of the biotin amplification immunohistochemistry protocol. Proteins are expressed in cells, with particular parts of them recognised as antigens. Specific primary antibodies then bind to these antigens. A biotin-labeled secondary antibody binds to the species-specific regions on the primary antibody and an avidin/biotin/ enzyme complex (ABC) associates itself with the conjugated biotin. The resulting enzymatic reaction with the chromagenic substrate creates a visible colour change thus localizing the site of the antigen expression.

2.6.1.1.7 Antigen detection

Antibody localisation was determined using 3,3'-diaminobenzidine (liquid DAB⁺; DAKO). DAB chromagen was diluted in the specific buffer, according to manufacturers' instructions, just prior to use. Sections were incubated with DAB until staining was optimally detected in control sections: this varied depending on the antibody and target tissue. The reaction was

stopped by immersing the sections in distilled water. A colour was generated at the site of the original antigen thus allowing its localisation within the tissue.

2.6.1.1.8 H & E counterstain and mount

Sections were lightly counterstained in Harris's haematoxylin, to stain cell nuclei blue, as described above (section 2.5). Slides were rinsed in tap water, dehydrated by immersing in 70% ethanol for 20 s, 85% ethanol for 20 s, 95% ethanol for 20 s, 100% ethanol for 20 s x2. Slides were then immersed in HistoClear™ (R A Lamb Limited, Eastbourne, UK) for 5 min and finally cleared in Xylene for 5 min. After immunostaining, glass coverslips (VWR, Lutterworth, UK; 22x32mmx1, Cat No.: 631-0133) were mounted on the sections using Pertex (Cell Path, Hemel Hempstead, UK), a solvent based glue. Slides were allowed to dry prior to archiving and storage in a dry, room temperature environment.

2.6.1.1.9 Light microscopy and Provis

Cellular sites of expression of target proteins were determined and slides photographed using a Provis AX70 (Olympus Optical, London, UK) microscope fitted with a Canon DS6031 camera (Canon Europe; Amsterdam).

2.6.1.2 Automated immunohistochemistry

An automated immunostaining machine was utilised to allow high throughput staining in a controlled and repeatable manner. The Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK) permitted staining of 30 slides per run. Exact conditions were optimised for each antibody and all kits were purchased from Vision Biosystems. Sections were deparaffinised, rehydrated (section 2.6.1.1.2) and antigen retrieval (section 2.6.1.1.3) performed as detailed above. A specific polymer high contrast programme was used for all antibodies, which applied the same principles as described above (section 2.6.1). Briefly, slides were peroxidase blocked for 5 min, incubated for 2 h with the primary antibody diluted to the optimal concentration in the diluent supplied and then incubated with the post-primary reagent for 15 min. Control sections were incubated with diluent alone to confirm antibody specificity. Sections were then incubated with the polymer reagent for 15 min to increase sensitivity of detection prior to DAB detection for 10 min. Sections were counterstained in haematoxylin for 5 min. Slides were then removed from the machine and dehydrated and mounted as detailed above (section 2.6.1.1.8).

2.6.1.3 Fluorescent immunohistochemistry

Fluorescent immunohistochemistry allows better visualisation of some antigens and allowed co-localisation of more than one antigen simultaneously.

Sections were dewaxed, rehydrated and antigen retrieval performed as detailed above (section 2.6.1.1). Sections do not require a peroxidase block as no peroxidase reagents were used in the detection method. All washes were performed using PBS (Sigma) and serum block was diluted in PBS/BSA rather than TBS/BSA.

2.6.1.3.1 Primary Antibody

After incubation with blocking serum, sections were incubated overnight at 4°C with the primary antibody diluted in serum/PBS/BSA.

2.6.1.3.2 Secondary Antibody

After washing in PBS, sections were incubated with a secondary antibody: this varied between protocols and the fluorescent chromagens used are summarized in Table 2.3. Slides must be protected from the light once the fluorescent chromagen was added to the slides to prevent bleaching of the fluorescent signal.

2.6.1.3.2.1 Direct method

This was a single-step process whereby sections were incubated with a secondary antibody directly labelled with a fluorescent tag. For example, pan-cytokeratin localisation was detected by incubating sections for 1 h with goat anti-mouse secondary antibody directly conjugated with alexa fluor 488 (Molecular Probes, Poort Gebouw, Holland) diluted 1:200 in PBS to produce green fluorescence.

2.6.1.3.2.2 Indirect method

This was a two-step process allowing amplification of the immunofluorescence. Firstly, sections were incubated with biotin labelled secondary antibody, raised against a species-specific sequence on the arm of the primary antibody. This signal was then amplified and detected using a fluorescently labelled streptavidin complex which bound to the biotin. For example, AR immunostaining was detected by incubating sections with biotinylated goat anti-rabbit IgG secondary antibody (DAKO) diluted 1:500 in NGS/PBS/BSA for 30 min followed by incubation for 1 h with streptavidin-conjugated alexa 546 (Molecular Probes), producing red fluorescence.

2.6.1.3.3 Counterstain and mount

Slides were washed in PBS and counterstained by incubation with either To-Pro 3 (Molecular Probes) for 2 min diluted 1:2000 in PBS, or with DAPI (DAPI; Sigma) for 10 min diluted 1:1000 in PBS. Both stains result in a nuclear-specific blue fluorescent label. Slides were then washed in PBS and mounted in Mowiol mounting medium (Calbiochem; Lutterworth, UK).

2.6.1.3.4 Fluorescent Microscopy

Fluorescent images were captured using a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

Table 2.3 Summary of fluorescent chromagens

Fluorescent complex	Supplier	Dilution	Exposure duration	Excitation wavelength (nm)/ emitted light colour
Streptavidin-conjugated alexa 546	Molecular Probes	1:200 in PBS	1 h	546/red
Streptavidin conjugated alexa 488	Molecular Probes	1:200 in PBS	1 h	488/green
Goat anti Mouse Cy5	Amersham	1:60 in PBS	1 h	633/blue
Goat anti Mouse 488	Molecular Probes	1:200 in PBS	1 h	488/green
To-Pro 3	Molecular Probes	1:2000 in PBS	2 min	655/blue
DAPI	Sigma	1:1000 in PBS	10 min	633/blue

To ensure reproducibility of results and allow accurate comparison of immunostaining between treatment groups, sections from control and treated animals were processed in parallel on at least three occasions: sections from at least 3 animals in each treatment group were run on each occasion. Appropriate negative controls were included, whereby the primary antibody was replaced by blocking peptide when available or normal serum alone, to ensure that any staining observed was specific: none of the antibodies used showed other than minor non-specific staining.

2.6.2 Western blots

Western blotting allows the separation of a mixture of proteins according to their molecular size. This method exploits the protein electro-mobility through a gel with specific sized

pores. These proteins are transferred onto a membrane, which then are labelled with antibodies specific to the protein of interest. The antibody binding is then visualised and quantified, as detailed below. To ensure reproducibility of results, each blot was performed several times and protein was isolated from at least 3 WDs from 3 different litters each time.

2.6.2.1 Protein extraction

Lysis buffer was used to break open the tissue thus allowing cells to release their proteins. Whole cell protein was harvested from frozen tissue using RIPA lysis buffer (section 2.14). In order to prevent degradation, tissue was kept frozen on dry ice until the lysis buffer was added and protease inhibitors were added to the lysis buffer. SDS was added to break non-covalent bonds within proteins and to give each protein a negative charge to facilitate their separation in acrylamide gels. Samples were homogenized in 75 μ l RIPA buffer using a cordless handheld motor driven grinder (Sigma, Z35,997-1) with a pestle designed to fit the 1.5 ml tube (Sigma, Z35,996-3): this disrupted the tissue and thus release of the protein. The homogenate was then incubated on ice for 1 h prior to centrifugation at 2500 rpm for 10 min. The protein enriched supernatant was collected in a fresh 1.5 ml Eppendorf™ and stored at -80°C until required.

2.6.2.2 Protein quantification

In order to correct for differences in amounts of protein recovered from each sample, protein concentration was quantified for each sample. The protein concentration was determined using a Biorad BCA protein assay kit according to manufacturer's instructions (Biorad Laboratories; Hemel Hempstead, UK) using solutions of known concentration of BSA in RIPA to standardise. Alternatively, protein concentrations were determined using the Genequant RNA/DNA calculator (Pharmacia Biotech, San Francisco, UK) using solutions of known concentration of BSA in RIPA to standardise.

2.6.2.3 Electrophoresis in acrylamide gels

2.6.2.3.1 Preparing acrylamide gels

SDS gels were prepared in two parts: a large pored upper stacking gel that contained the wells and concentrated the sample prior to separation and a resolving gel below it with smaller pores. Resolving gel (5 ml; section 2.14) was pipetted into a plastic cassette (Invitrogen, Paisley, UK) and allowed to set at room temperature for 20 min. This was overlaid with distilled water to block out any air and so allow the gel to set. Once set, the water was poured off and the stacking gel (2 ml; section 2.14) was pipetted into the cassette on top of the resolving gel. A 10 or 15 well comb was slid into place, ensuring no air bubbles were present to interfere with efficient loading and running of the gel. Gels were left

to set then stored in a humid environment at 4°C. Gels were prepared with 7.5%, 10% or 12% weight/volume acrylamide to allow optimal separation of proteins: larger proteins pass through lower percentage gels easier and so faster while higher percentage gels allow protein molecules to run slower thus better separating out proteins that are of similar sizes.

2.6.2.3.2 Preparing samples

Optimally, 15 µg of protein was used but the amount of protein used in each Western blot was restricted by the sample availability, concentration and the finite volume of the wells in the gels.

Proteins were denatured by boiling with 5 µl 2x SDS loading buffer (1.0 ml glycerol, 0.5 ml β-mercaptoethanol, 3.0 ml 10% SDS, 1.25 ml 1.0M Tris-HCL pH6.7 and 1-2 mg bromophenol blue) for 5 min before loading each protein extract onto a cast polyacrylamide gel (7.5-12%). Protein samples were carefully loaded into the wells of the cast gel and 5 µl of a pre-stained multi-coloured standard for SDS-PAGE (Invitrogen, LC5725) was loaded in at least one lane per gel. This marker included proteins of known molecular weight thus allowing comparison against the expected position of the target protein.

2.6.2.3.3 Running gels

SDS-PAGE relies on proteins' ability to migrate through polyacrylamide gel pores, from the cathode to the anode, when under an electrical charge: proteins are treated with SDS to make them negatively charged. The gel was positioned in the tank with 1 x SDS running buffer (25mM Tris Base, 250mM Glycine and 0.1% SDS) and subjected to electrophoresis at 100V under reducing conditions until the pre-stained multi-coloured standard reached the bottom of the gel, approximately 1-2 h. Care was taken to ensure the proteins were not allowed to run too far through the gel and so lost off the bottom.

2.6.2.4 Western transfer of proteins

Having separated the proteins according to their molecular weight, they were transferred and immobilised to a nitrocellulose membrane. The protein gel plates were prised apart and the protein gel was carefully removed to a petri dish filled with running buffer until the blotting apparatus was assembled.

The nitrocellulose membrane (Immobilon-P; Millipore, Bedford, MA) was rehydrated in methanol for 30 s then washed in transfer buffer. The porous pads and 6 pieces of 3mm Whatman paper were also soaked in transfer buffer to prevent dehydration of the gel when assembling the blotting apparatus. A gel/membrane sandwich was assembled according to manufacturers' instructions as shown in Figure 2.3, and placed in a transfer tank. This

arrangement of the gel and the membrane allows the SDS treated proteins to be transferred laterally from the gel towards the anode until their migration was stopped by the membrane. The tank was filled with a methanol transfer buffer (in-house protocol, see below) and attached to a power pack. The blot was electro-transferred for 4 h at 40V or overnight at 20V.

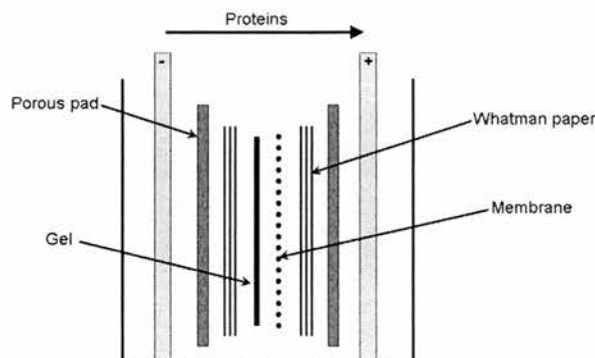


Figure 2.3 Schematic representation of Western blot apparatus

2.6.2.5 Protein detection

The Western blot apparatus was dismantled and the membranes were washed in TBS containing 0.1% Tween 20 (TBST; Sigma). Antibodies were then used to detect specific proteins on the membrane.

Two analysis systems were available for detection of the proteins, the Typhoon 9400 variable mode imager (Amersham Bioscience), which utilised a horseradish peroxidase detection system or the Li-Cor (Li-Cor, Nebraska, USA) which detected the proteins using fluorescence. The Li-Cor system allows the detection of more than one protein simultaneously but was not available until my final year, therefore initial Western blot analysis was carried out on the Typhoon.

2.6.2.5.1 Analysis on Typhoon

2.6.2.5.1.1 Blocking of non-specific binding

As with immunohistochemistry, non-specific binding sites had to be blocked. This was done by incubating membranes in TBST containing 5% (weight/volume) powder milk (shop bought) for 1 h at room temperature.

2.6.2.5.1.2 Primary antibody

Membranes were washed twice in TBST for 5 min then incubated overnight at 4°C with primary antibody. The same primary antibodies were used as for immunohistochemistry

(Table 2.1) but were diluted in TBST 10x more than for immunohistochemistry. An anti- β -tubulin antibody (Sigma) was used as a standardization control, diluted 1:300 in TBST.

2.6.2.5.1.3 Secondary antibody

The secondary antibodies used were directed against the primary antibody host species, as for immunohistochemistry (section 2.6.1.1.6), and were conjugated with HRP. The HRP reacted with the chemiluminescent substrate to create light emission at the site of antigen expression. The specific pattern of light emission was captured by timed exposure to photosensitive film in a dark room.

After washing off any residual primary antibody with TBST for 5 min (x3), membranes were incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase or anti-mouse horseradish peroxidase labelled secondary antibody (Scottish Antibody Production Unit, Carlisle, UK) diluted 1:5000 in TBST/5% milk.

2.6.2.5.1.4 Visualisation of proteins

Membranes were again washed in TBST for 5 min x3 prior to detecting the signal using Enhanced Chemi-luminescence (ECL plus™, Amersham Biosciences, Buckinghamshire, UK) Western blot detection reagents according to manufacturers instructions (Amersham Biosciences). Signals were visualized using high performance chemi-luminescence imaging film (Amersham Bioscience) and developed using a Xograph compact X4 imaging system (Xograph, Tetbury, UK). Exposure times were optimised for each experiment according to the intensity of the signal detected. Antibody specificity was confirmed by the detection of only one band at the expected size when using each antibody. The intensity of the bands was then quantified using the Typhoon 9400 variable mode imager (Amersham Bioscience). The image was analysed using the ImageQuant software (Amersham Life Sciences, UK) and the area of film exposed to the chemiluminescence was quantified per gel, per antibody and per sample. The area of exposure equated to the amount of labelled protein present in the sample. The protein expression level was corrected for loading using β -tubulin and related to e19.5 control protein levels.

2.6.2.5.1.5 Stripping of blots

Bound antibodies can be stripped from Western blot membranes, leaving the transferred proteins intact, to permit hybridisation with another antibody. This allowed quantification of several proteins on one blot and allowed different protein expressions to be quantified against each other. This was particularly useful as WDs only yielded small amounts of protein. Blots could be stripped and re-probed successfully up to 3 times, although care was

taken to avoid using more than one primary antibody that had been raised in the same species to minimise background staining. Restore Western Blot Stripping buffer TM (Pierce; supplied by Perbio Ltd, Northumberland, UK) was added to the membrane for 1 h at room temperature. To check for complete stripping, the membrane was then washed in TBST before incubation with the secondary antibody against the primary antibody being stripped. The membrane was again washed in TBST then developed using ECL plusTM. No bands should be detected.

2.6.2.5.2 Western blot analysis using the Li-Cor

2.6.2.5.2.1 Blocking

Non-specific binding sites were blocked by incubating membranes for 1 h at room temperature in Odyssey® blocking buffer (Li-Cor, Nebraska, USA) diluted 1:1 in PBS.

2.6.2.5.2.2 Primary antibody

Membranes were incubated overnight at 4°C with the primary antibody, diluted in Odyssey® blocking buffer/PBST. Tween was added to the PBS in order to reduce any background. The same primary antibodies were used as for immunohistochemistry (Table 2.1) but were diluted 10x compared with the dilution used for immunohistochemistry. An anti-β-tubulin antibody (anti-mouse, Sigma; anti-rabbit, Santa Cruz) was used as a standardization control, diluted 1:300.

The Li-Cor allows detection of more than one antibody at a time, therefore 2 antibodies could be added at once so long as their host species differed so they could be discriminated by secondary antibodies of different specificities. Before combining 2 primary antibodies, preliminary blots were performed using each primary antibody alone to confirm the expected band pattern and eliminate any possible background bands. Both primary antibodies were diluted, at the appropriate dilution, in the Odyssey® blocking buffer/PBST and incubated with the membrane simultaneously for 1 h at room temperature.

2.6.2.5.2.3 Secondary antibody

The secondary antibodies used were directed against the primary antibody host species, as for immunohistochemistry (section 2.6.1.1.6), and were fluorescently labelled. Membranes and reagents were therefore protected from light from this step onwards.

Residual primary antibody was washed off with PBST for 5 min (x3). Membranes were incubated for 1 h at room temperature with the appropriate secondary antibody (Table 2.5) diluted 1:5000 in Odyssey® blocking buffer/PBST. If two-colour detection of two primary

antibodies was used, both secondary antibodies were added to the membrane simultaneously. For example, if detecting AR (rabbit) and SMA (mouse) simultaneously, goat anti-rabbit 800 and goat anti-mouse 680 secondary antibodies were added. AR protein would be indicated by the presence of red bands while SMA would be shown as green bands on the membrane when visualised with the Li-Cor.

Table 2.4 Summary of secondary antibodies for Western blot detection using the Li-Cor.

Fluorescent complex	Species	Supplier	Excitation wavelength (nm)/ emitted light colour
Alexa Fluor ® 680	Donkey anti goat	Molecular Probes	680/green
Alexa Fluor ® 680	Goat anti mouse	Molecular Probes	680/green
IRDye™ 800 conjugated	Rabbit anti goat	Rockland (Gibertsville, USA)	800/red

2.6.2.5.2.4 Visualisation of proteins

Membranes were again washed in PBST for 5 min (x3) then PBS (x1), to remove any residual Tween, prior to detecting the signal. The membrane was scanned using the Li-Cor, according to manufacturers’ instructions. Antibody specificity was confirmed by the detection of only one band at the expected size when visualising each antibody. The intensity of the bands was then quantified, with the area of exposure equating to the amount of labelled protein present in the sample. If two-colour detection was used, quantification was carried out for each antibody separately. The protein expression level was corrected for loading using β -tubulin and related to e19.5 control protein levels.

2.6.2.5.2.5 Stripping the membrane

Bound antibodies were stripped from Western blot membranes after developing to permit hybridisation with another antibody. Blots could be stripped and re-probed successfully up to 2 times, although care was taken to avoid overstripping the membrane as this can remove target proteins. Stripping buffer (25nM glycine pH2.0 + 1-2% SDS) was added to the membrane for 10-15 min at room temperature. To check for complete stripping, the membrane was re-scanned to confirm no bands were present. Generally, stripping was not particularly successful with this detection method and was rarely used.

2.7 RNA analysis

2.7.1 RNA extraction

RNA was isolated from frozen tissues using the RNeasy Mini extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Briefly, samples were homogenised in the supplied lysis buffer using a cordless handheld motor driven grinder (Sigma, Z35,997-1) with a pestle designed to fit the 1.5 ml tube (Sigma, Z35,996-3): this facilitated the disruption of the tissue and thus release of the RNA. Any genomic DNA contamination was removed from the sample by DNase treatment during extraction using RNase-free DNase on the column digestion kit (Qiagen) according to manufacturers' instructions.

2.7.2 RNA quantification

In order to correct for differences in amounts of RNA recovered from each sample, RNA concentration was quantified using RNA 6000 Nano chips on an Agilent 2100 Bioanalyser (Agilent Technologies, Wokingham, UK). This allowed quantification of both RNA concentration and integrity.

2.7.3 Reverse transcription

Reverse transcription of RNA to cDNA was primed with oligo-dTs or random hexamers, depending on the final application to be used. Oligo-dTs are specific to mRNAs with a poly A⁺ tail therefore ensuring amplification from mRNA only, however, they do not always amplify up the 5' end of long mRNAs. Random Hexamers amplify total RNA therefore they must be used to amplify ribosomal RNA, vital for Taqman reactions since 18S, a ribosomal RNA, is used as the internal positive control. Random hexamer primed reverse transcription will also yield more cDNA.

2.7.3.1 Oligo-dTs

Oligo-dT primed cDNA was amplified using Bioscript Reverse Transcriptase (Bioline, Germany) according to manufacturer's instructions, as detailed below.

Total RNA	1 µg
Oligo dT (0.5 µg/µl) (Invitrogen)	1 µl
Water	<u>up to 11 µl</u>
Total	12 µl

Samples were incubated at 70 °C for 5 min then chilled on ice before adding 8 µl of the following mastermix.

Mastermix:

	Actual volume	Final Concentration
RNase inhibitor (20units/µl, Promega)	0.5 µl	0.5unit/µl
10mM dNTPs (Promega)	0.4 µl	0.2mM
5xRT Buffer	4 µl	1x
Water	2.85 µl	
Bioscript Reverse Transcriptase	<u>0.25 µl</u>	0.625unit/µl
	8 µl per reaction	

Total final reaction volume = 20 µl

Samples were incubated at 42°C for 1 h before stopping the reaction by heat inactivating the enzyme at 70°C for 10 min.

2.7.3.2 Random Hexamers

Random hexamer primed cDNA was prepared using the Applied Biosystems TaqMan ® reverse transcription kit (Applied Biosystems, Foster City, CA) according to manufacturers' instructions.

Mastermix:

	Actual volume	Final Concentration
Water	1.85 µl	
10x RT buffer	1 µl	1x
25nM MgCl ₂	2.2 µl	6.25mM
2.5mM dNTP	2 µl	0.5mM
Random Hexamers (50µM)	0.5 µl	2.5µM
RNase inhibitors (20U/µl)	0.2 µl	0.5 U/µl
Reverse Transcriptase (50U/µl)	0.25 µl	1.25 U/µl
RNA (100ngµl)	<u>2 µl</u>	
Total	10 µl	

The reagents were added together in a 0.2 ml sterile thin walled PCR tube (Continental Lab Products, Oxford,UK) and cycled in a thermo-cycler through the following programme: 25°C for 20 min, 42°C for 60 min and 95°C for 5 min.

2.7.4 Taqman ® PCR

Quantitative PCR was performed with the ABI Prism 7900 Sequence Detection System (Applied Biosystems). TaqMan ® uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. Expression of specific gene mRNA was determined using the Assay-On-Demand Gene Expression™ system (Applied Biosystems), as detailed in Table 2.5 below. All reagents were obtained from Applied Biosystems, unless otherwise stated. Samples were run in triplicate on a 96 well MicroAmp optical reaction plate (Applied Biosystems). The expression level of the specific gene mRNA was related to an internal control, 18S ribosomal RNA. Appropriate positive and negative controls were included in each plate. Results shown are the mean of a minimum of 3 WDs per treatment group performed in triplicate on at least 2 occasions.

For each sample the following mastermix was prepared and 25 µl was aliquoted into each well. The plate was sealed with ABI prism optical adhesive optical cover (Applied Biosystems) and loaded onto the ABI 7900 HT sequence detection system.

Mastermix:

	Actual volume	Final Concentration
2x PCR mastermix	37.5 µl	1x
Assay on demand primer/probe	3.75 µl	1x
18S primer/probe mix	1.125 µl	0.02µM (primer) 0.08µM (probe)
Water	25.125 µl	
cDNA	<u>7.5 µl</u>	
Total	75 µl	

Table 2.5 Summary of Taqman ® Assay-On-Demand Gene Expression™ primers used

Gene of interest	Assay number
AR	Rn00560747
SMA	Rn00563662
MMP-2	Rn01538167
IGF-1	Rn0710306

2.7.4.1 Analysis of results

Taqman PCR results are displayed as an amplification plot, which shows the amount of the reporter dye generated during amplification: this relates directly to the amount of PCR product formed and thus the amount of target gene expressed in the target tissue. When analysing the data, the baseline value and threshold must all be considered. The baseline value refers to basal fluorescence in the reaction plate while the threshold value is the point at which an increase in signal is associated with an exponential increase of PCR product. This threshold value should therefore be set in the exponential region of the amplification plot rather than in the plateau or baseline regions. The ct (threshold cycle) reflects the cycle number where the fluorescence that is generated in a reaction crosses the threshold. This ct value reflects the cycle number at which the level fluorescence due to amplification of the PCR product reaches a significant level above the background fluorescence (threshold level) (Fig 2.4).

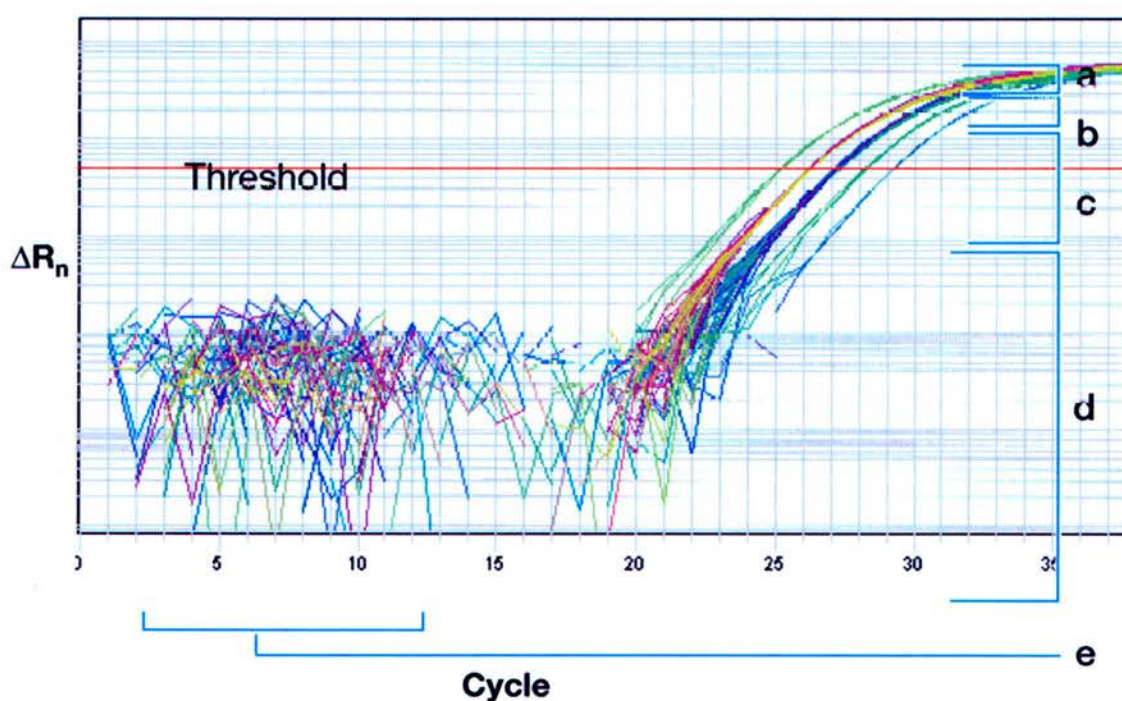


Figure 2.4 A typical amplification curve after a successful Taqman run. a - plateau phase, b - linear phase, c - exponential phase, d - background, e – baseline. (Adapted from Applied Biosystems, Relative Quantitation Using Comparative CT - Getting Started Guide).

A relative quantitation assay is used to analyse any changes in gene expression in a given sample, relative to another reference sample (such as an untreated control sample). This relative quantification is achieved using the comparative ct method, which utilizes arithmetic formulas. For each sample the ΔCT is calculated by subtracting the ct for the internal

positive control gene from the ct for the target gene; e.g. AR ct – 18S ct. This is then averaged for the triplicate for each sample and is then compared to a reference sample. This is calculated by subtracting the Δ CT for the reference sample from the Δ CT for the target sample and is referred to as $\Delta\Delta$ CT. An arithmetic formula is then applied to calculate the fold change in the target gene in the target sample compared to the reference sample: $2^{-\Delta\Delta$ CT. Values were expressed as a fold difference relative to uterus, unless otherwise stated.

2.8 Luminal length analysis

Differentiation of WDs was quantified by measuring the luminal length of the epididymal segment of WDs from control and treated animals: a line was digitally drawn through the lumen of the WD image taken at time of dissection using the Image J program (National Institute of Health, USA). This line was drawn from the head of the epididymis, where the efferent ducts end, to the tail of the epididymal section just prior to the start of the vas deferens. To ensure reproducibility and to correct for individual variation, luminal length was measured for WDs from 15-37 animals from at least 3 different litters per treatment group. Comparisons were then made between control and treatment groups.

Only samples that were analysable were studied: for example, WDs from flutamide-exposed animals with incomplete lumens (7% of animals at e21.5) were not included in the analysis of luminal length. Thus, results presented for luminal length are likely to underestimate the overall effect of flutamide exposure.

2.9 Epithelial height and width measurements

WD sections were immunostained for pan-cytokeratin as detailed above to label clearly all epithelial cells. Sections were viewed using the x63 objective on an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Image-Pro Plus version 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) was utilized to measure epithelial cell height and width. Using a x63 objective, epithelial cell height and width were measured in every 5th epithelial cell per section. Height measurements were performed separately for the caput, corpus and cauda regions of the future epididymal portion of the WD. Only epithelial cells in which the nucleus could be clearly identified were measured, thus excluding from analysis any epithelial cells from the flutamide treatment groups that were severely flattened or disintegrating.

2.10 Apoptosis analysis

Cleaved caspase 3 immunostaining was performed on WDs from control and flutamide exposed fetuses using standard methods, as detailed above, to stain for apoptotic cells. Very few cleaved caspase 3 positive cells were detected, therefore a detailed stereological analysis was not appropriate and all positive cells were manually counted in each WD using an Axiolab microscope (Carl Zeiss Ltd, Hertfordshire, UK).

2.11 Statistical Analysis

Where appropriate, values were expressed as Means \pm S.E.M. Differences between means were analysed using an unpaired two-tailed t-test (for 2 groups) or one-way Analysis of Variance (ANOVA) followed by the Bonferroni post-hoc test (for 3 or more groups). The criterion for significance for all tests was set at $p < 0.05$. All analyses were carried out using GraphPad Prism version 4 (Graph Pad Software Inc., San Diego, CA, USA).

2.12 Commonly used solutions

2.12.1 Fixatives

2.12.1.1 Bouin's Soution

Picric acid can no longer be bought except by industry so Bouin's solution was made for us to this recipe by Triangle Biomedical Sciences Ltd, Lancashire, UK.

2.12.1.2 4% (w/v) Paraformaldehyde

Paraformaldehyde (Sigma)	4 g
Distilled water	50 ml

The PFA solution was heated to 60°C until the PFA depolymerised and dissolved: the solution was not allowed to heat above 70°C. 1-2 drops of 1M NaOH was added to clear the solution. This 8% (w/v) PFA in distilled water was then diluted to 4% in PBS by cooling the solution to room temperature and adding 50 ml 2M PBS (Sigma). This 4% PFA solution was filtered and the pH was checked to be between pH7.2 - 7.4.

PFA was used on the day of making or frozen in aliquots at -20°C.

2.12.2 Stains

2.12.2.1 Harris's Haematoxylin

Haematoxylin (2.5g) was dissolved in Absolute Alcohol (25 ml). Aluminium Potassium Sulphate (50g) was dissolved in 500 ml warm distilled water and the haematoxylin solution

was added. The solution was brought to the boil and mercuric oxide (1.25g) was added slowly. The haematoxylin solution was plunged into ice to cool it before filtering it into a staining dish. Glacial acetic acid (4 ml per 100 ml Haematoxylin) was added.

2.12.2.2 Eosin

An aqueous eosin solution was made by dissolving 1% (w/v) eosin (15 g) in water (1500 ml). An alcohol solution was also made by dissolving 1% (w/v) eosin (5 g) in methylated spirit (500 ml). Both solutions were mixed together (3 parts aqueous to 1 part spirit eosin solution) and filtered. Formaldehyde (1 ml) was added to prevent bacterial growth and a sprinkle of calcium chloride was added.

2.12.2.3 Acid Alcohol

70% Ethanol

1% Concentrated hydrochloric acid

2.12.2.4 Scotts Tap Water

Potassium hydrogen carbonate	10 g
Magnesium sulphate	100 g
Tap water	5 l

2.12.3 Common Buffers

2.12.3.1 Tris buffered saline (TBS) 0.5M

Tris (Sigma)	60.5 g
NaCl	87.6 g
HCl	300 ml
Water	<u>up to 10 l</u>
Total	10 l

The solution was corrected to pH7.4 using concentrated HCl.

This 10x TBS solution was diluted to 1x TBS using distilled water before use.

2.12.3.2 EDTA 0.5M

EDTA (Sigma)	186.1 g
Distilled water	800 ml

The solution was corrected to pH 8.0 with NaOH then made up to 1 l with distilled water.

2.12.3.3 TE buffer 1x

1M Tris	5 ml
0.5M EDTA	1 ml
Water	<u>494 ml</u>
Total	500 ml

2.12.3.4 Tris-Acetate-EDTA (TAE) Buffer (50x)

Tris (Sigma)	242 g
Acetic acid (BDH)	57.1 ml
0.5M EDTA	100 ml
Water	<u>up to 1 l</u>
Total Volume	1 l

The pH of the solution was adjusted to pH8.5. This stock solution was diluted to 1x with distilled water before use.

2.12.3.5 RIPA Protein Extraction Lysis buffer

	Final Concentration
Triton X-100	1%
HEPES-NaOH (pH 7.5)	15 mM
NaCl	0.15 mM
Sodium deoxycholate	1%
Sodium dodecyl sulfate (SDS)	0.1%
Sodium orthovanadate	1 mM
EDTA	10 mM
Protease inhibitor cocktail (Sigma)	0.5%

2.12.3.6 Western blot Transfer buffer

Methanol	100 ml
Glycine	7.2 g
Tris,	1.53 g
Distilled water	<u>up to 1 l</u>
Total	1 l

2.12.4 Acrylamide gels

4% (w/v) Stacking gels:

Water	3.62 ml
30% Acrylamide	0.65 ml
1.0M Tris pH6.8	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	<u>0.005 ml</u>
Total	5 ml

Resolving gels:	7.5%	10%	12%
Water	7.19	5.94	4.94
30% Acrylamide	3.75	5.0	6.0
1.5M Tris pH8.8	3.8	3.8	3.8
10% SDS	0.15	0.15	0.15
10% APS	0.1	0.1	0.1
TEMED	<u>0.01</u>	<u>0.01</u>	<u>0.01</u>
Total	15 ml	15 ml	15 ml

3 The impact of androgen action on normal Wolffian duct development

3.1 Introduction

It is widely accepted that sexual differentiation of the male reproductive tract is dependent on hormone action (George, 1994). For example, the Wolffian duct (WD) is pre-programmed to regress and degenerate unless it receives a signal to initiate rescue and stabilisation. This signal is believed to be testosterone, which is secreted by the fetal testis from e15 in rats (Warren *et al*, 1972). Once stabilised, the male WD begins to differentiate into its adult derivatives: the cranial portion convolutes to form the epididymis, the central portion remains a simple straight duct but develops a thick muscular coat to form the vas deferens, while the seminal vesicles bud off the distal segment (Robaire, 1988). By e21.5 in rats, this segmentation of the WD is obvious with the future epididymal segment already highly convoluted at this age (N.Marchetti, unpublished thesis).

Previous researchers have shown that interfering with androgen action during fetal life, either using androgen receptor mutant animals or exposure to anti-androgenic compounds, results in abnormal reproductive tracts in adult males (reviewed in Lyon and Hawkes, 1970; reviewed in Gray *et al*, 2001; Yeh *et al*, 2002) (section 1.5.2). Most studies have exposed rats to anti-androgenic compounds between e13-21, as this encompasses the onset of testosterone production by the testis and the window of male reproductive development, and have then examined the effects on the adult reproductive tract. In these adult males, the epididymis and/or vas deferens is often incomplete or absent altogether: it is generally presumed that this is due to a failure of the male WD to stabilise early in fetal development, however, this has not been directly demonstrated since few of these researchers have examined the morphology of the fetal WD in any detail (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1998; McIntyre *et al*, 2000). The adult phenotype could in fact be due to degeneration of the WD-derivatives later in life. Therefore further detailed investigation into the morphology of the fetal WD is required.

Various compounds have been utilised to examine the effects of reduced androgen action on WD development including linuron, vinclozolin, phthalates and flutamide (section 1.5.2.2). Flutamide is one of the most potent anti-androgens as it is a highly competitive androgen receptor antagonist and it induces a high prevalence of reproductive tract abnormalities in males without inducing any obvious toxicity (Imperato-McGinley *et al*, 1992). Flutamide was developed by the pharmaceutical industry specifically as an AR antagonist therefore its mode of action is specific and well characterised, unlike many other anti-androgens which

were developed for other functions such as vinclozolin, a fungicide that happens to have anti-androgenic properties (section 1.5.2.2). Unlike phthalates, flutamide acts on the AR and does not reduce testosterone production, therefore any impact on WD development can be directly correlated with reduced androgen action (Xu and Li, 1998; Fisher *et al*, 2003).

Administration of flutamide to pregnant rats between e14.5-21.5 has been shown to dose-dependently impair masculinisation. For example, treatment with 18mgkg⁻¹ flutamide resulted in complete feminisation of external genitalia but normal WD differentiation in male offspring whereas at doses above 100 mgkg⁻¹ flutamide, the vas deferens was absent unilaterally or bilaterally and only small remnants of the epididymis were present in adults (Imperato-McGinley *et al*, 1992). To the best of my knowledge, no such studies have examined the phenotype and morphology of the fetal WD or the mechanisms involved in the degeneration of WD-derived tissues by adulthood.

Whilst it is obvious that androgens play a role in WD development, the molecular and cellular events that underlie its stabilisation and subsequent differentiation have received little attention to date. To address this, we have used a model system in which WD development can be altered, thus enabling the investigation of WD stabilisation and differentiation with the aim of elucidating the cellular mechanisms responsible for androgen dependent WD development.

3.1.1 Aims

To establish a model for manipulating androgen action on the fetal urogenital tract in our rat colony, comparable to models previously published, using maternal flutamide exposure.

To investigate the effect of maternal flutamide exposure on the WD. To establish if flutamide-induced blockade of androgen action interferes with stabilisation of the male WD, as occurs naturally in females, and/or its differentiation.

To investigate whether androgens control WD development by altering cell proliferation and/or apoptosis and if this preferentially targets one cell compartment.

3.2 Materials and Methods

3.2.1 *In utero* treatments

As detailed in section 2.2, pregnant dams were randomly allocated to treatment groups and dosed once daily by gavage with 1 ml kg⁻¹ maternal bodyweight of the various treatments detailed below. Dams were dosed between e15.5-e21.5, or until the day prior to cull if this is before e21.5. This window of exposure was selected to encompass the onset of androgen action at e15.5 and the period of fetal male reproductive development e15.5-21.5 according to (Warren *et al*, 1972).

Flutamide: 50 mg kg⁻¹ in 1ml corn oil/2.5% DMSO (n=24 litters)
 100 mg kg⁻¹ in 1ml corn oil/2.5% DMSO (n=23 litters)
Control: 1ml corn oil/2.5% DMSO (n=68 litters)

These doses were selected based on previous results which reported a high incidence of male reproductive tract malformations in rats without any adverse toxicological effects (Imperato-McGinley *et al*, 1992).

Throughout this chapter, offspring from dams treated with flutamide while pregnant will be referred to as “flutamide-exposed” or treated “*in utero*”. This does not mean that fetuses were directly exposed to flutamide but were exposed indirectly via their mother.

3.2.2 Tissue collection

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation and fetuses were recovered at e17.5 – e21.5. Postnatal animals, older than pnd10, were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Fetuses (e21.5) and postnatal animals were weighed and anogenital distance measured as described in section 2.3.

Fetuses were killed by decapitation then reproductive tracts were recovered by microdissection and photographed using a Leica ICA camera (section 2.3.2.1). Reproductive tracts and gonads were collected from postnatal animals and photographed as described in section 2.3.2.2. Reproductive tracts were analysed microscopically, at the time of dissection, for any gross morphological abnormalities. Tissue was either fixed or frozen as detailed in section 2.4.

3.2.3 Luminal length analysis

Differentiation of fetal and neonatal WDs was quantified by digitally measuring the luminal length of the epididymal segment of WDs from control and treated animals, as described in chapter 2, section 2.8. Only samples that were analysable were studied: for example, WDs from flutamide-exposed animals with incomplete lumens (7% of animals at e21.5) were not included in the analysis of luminal length. Thus, results presented for luminal length are likely to underestimate the overall effect of flutamide exposure.

3.2.4 Histological analysis

Most histological analyses were performed on WD sections stained with haematoxylin and eosin, using standard protocols as detailed in chapter 2, section 2.5. WDs were inspected carefully and note was taken of any abnormalities seen.

3.2.5 Co-immunoexpression of epithelial and stromal compartments

In order to delineate stromal and epithelial compartments, fluorescent immunohistochemistry was used to co-localise cell-specific proteins in WDs recovered from control and flutamide-exposed fetuses at e17.5 – e21.5. Sections were deparaffinised, rehydrated and antigen retrieval performed as detailed in section 2.6.1. All washes between antibody or reagent incubations comprised two 5 min washes at room temperature in PBS (Sigma). At each stage, control sections were incubated with blocking serum without antibody in order to confirm antibody specificity. Non-specific binding sites were blocked by incubating sections in normal goat serum (NGS; Autogen Bioclear UK Ltd, Wiltshire, UK) diluted 1:4 in PBS containing 5% bovine serum albumin (BSA; Sigma). Sections were incubated overnight at 4°C with anti-pan-cytokeratin antibody (Sigma) diluted 1:200 in NGS/PBS/BSA. Pan-cytokeratin localisation was detected by incubating sections for 1 h with goat anti-mouse secondary antibody directly conjugated with alexa fluor 488 (Molecular Probes) diluted 1:200 in PBS to produce green fluorescence. Sections were incubated for 30 min with mouse IgG (Sigma) diluted 1:2000 in NGS/PBS/BSA to block any remaining mouse IgG sites and prevent the second mouse antibody sticking non-specifically. Non-specific binding sites were blocked again by incubating sections with NGS/PBS/BSA for 30 min before incubating overnight at 4°C with anti-smooth muscle actin antibody (SMA; Sigma) diluted 1:500 in NGS/PBS/BSA. Sections were incubated for 1 h with goat anti-mouse labelled with Cy5 (Amersham Biosciences, Little Chalfont, UK) diluted 1:60 in PBS, producing blue fluorescent SMA immunostaining. Sections were counterstained for 10

min with a nuclear-specific blue fluorescent label (DAPI; Sigma) diluted 1:1000 in PBS. Slides were then washed in PBS and mounted in Mowiol mounting medium (Calbiochem; Lutterworth, UK). Fluorescent images were captured using a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

3.2.6 Epithelial height and width measurements

WD sections were immunostained for pan-cytokeratin (section 2.6.1) to label clearly all epithelial cells and epithelial cell height and width were measured in every 5th epithelial cell in the future epididymal segment, as detailed in section 2.9.

3.2.7 Measurement of WD width

WD sections were stained by H&E (see section 2.5) and viewed using the x63 objective on an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Image-Pro Plus version 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) was utilized to measure the width of the WD in at least 3 evenly spaced places along the length of the epididymal section.

3.2.8 Frequency of cell mitoses in WDs

In order to determine if cell proliferation in WD compartments was affected by flutamide treatment, various cell cycle markers and analytical methods were investigated. For technical reasons, it was considered that determination of the proliferation index was impractical for the densely packed stromal cell compartment: previous researchers have commented on the difficulty of counting cellular ratios in the WD (Bowman *et al*, 2005). Instead, a method was devised to allow enumeration of the total number of mitotic cells in each compartment, as outlined below.

3.2.8.1 Immunostaining of mitotic cells

WD sections from control and flutamide exposed fetuses at e19.5 – e21.5 were immunostained for phospho-histone H3 (Upstate Biotechnology, Dundee, UK), a mitotic marker, using a Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK) and a polymer high contrast programme. Briefly, after high pressure antigen retrieval, slides were peroxidase blocked for 5 min, incubated for 2 h with the primary antibody diluted 1:1000 in the diluent supplied and then with the post-primary reagent for 15 min. Control sections were incubated with diluent alone to confirm antibody

specificity. Sections were then incubated with the polymer reagent for 15 min to increase sensitivity of detection prior to DAB detection for 10 min, counterstained in haematoxylin for 5 min, dehydrated and mounted.

3.2.8.2 Analysis of mitotic cells

Phospho-histone H3 positive cells were counted in the epithelial compartment and in the inner and outer stromal layers of the WD (Fig. 3.1) using the x20 objective on an Olympus BH-2 microscope fitted with a Prior automatic stage.

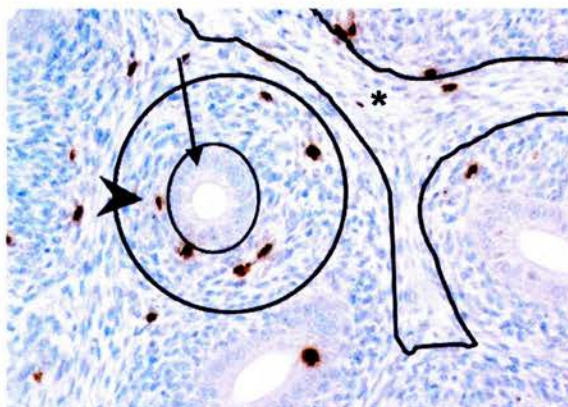


Figure 3.1 Image of e21.5 WD stained for phospho-Histone H3 (brown) to identify mitotic cells. Note that examples of the different cellular compartments are delineated by black lines and mitotic cells (brown) were counted in each compartment individually. Epithelium (arrow), inner stroma (arrowhead) and outer stroma (*).

Image-Pro Plus version 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK) was utilized for analysis of the number of mitotic cells. Positive cells were only counted in the future epididymal portion of the WD, not in the efferent ducts or vas deferens. Since differences were noted in the degree of coiling along the length of the future epididymal portion of the WD, phospho-histone H3 positive cells were initially counted in each region of the future epididymis individually, defined as in the adult epididymis as caput, corpus and cauda (Robaire, 1988). However, no consistent difference was seen in phospho-histone H3 staining between the different regions of the epididymal portion of the WD, therefore proliferation was subsequently analysed in the epididymal portion of the WD as a whole.

Variation was noted in the amount of the WD visible in each section as well as the amount of epithelium visible per section: this was due to both treatment effects and the plane of sectioning. To correct for this variation, the overall length of the WD and the length of epithelium visible in each section were measured. The number of phospho-histone H3 positive cells in the epithelial compartment was then divided by the total length of

epithelium visible in the section to calculate proliferation per micron of epithelium (see Fig. 3.2). Since flutamide exposure reduces coiling and WD luminal length, it was important to take this into account when analysing cell proliferation. The number of positive epithelial cells per micron of epithelium visible was therefore multiplied by the luminal length for that specific WD (measured at the time of dissection, method detailed above) in order to calculate total epithelial cell proliferation in the whole epididymal portion of the WD, not just cell proliferation in the section being analysed. Similarly, the number of phospho-histone H3 positive cells in the stromal compartment was divided by the length of WD epithelium visible in to correct for variation in the length of the WD. This was then multiplied by the luminal length of the WD to calculate total stromal proliferation in the whole epididymal portion of the WD. Sections from 8-14 fetuses from 3-5 separate litters were analysed from both control and flutamide-exposed animals at each age.

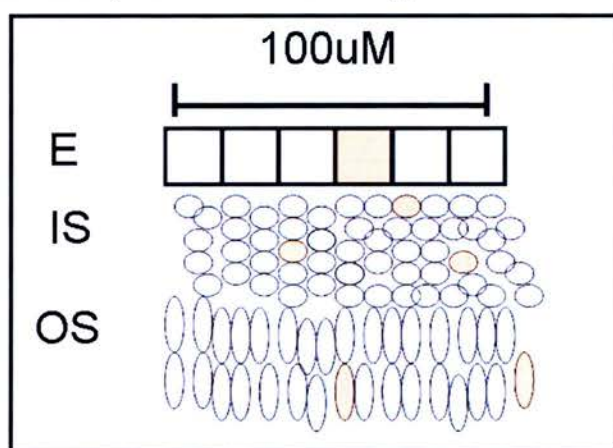


Figure 3.2 Diagrammatic representation of the proliferation analysis method. Mitotic cells (orange) were counted in each cellular compartment (E – epithelium, IS – inner stroma, OS – outer stroma). The length of epithelium visible in each section was measured and the number of positive cells was corrected to this.

3.2.8.3 Epithelial mitotic index

To confirm that this analysis was likely to reflect the cell proliferation/mitotic index, the total number of phospho-histone H3 positive and negative epithelial cells were counted (500-700 cells) in 3 control and 3 F100 e21.5 WDs, and an epithelial mitotic index was derived (phospho-histone H3 positive cells/total number of cells x100%). This was not able to be calculated for the stromal compartment as it was technically impossible to count individual stromal cells.

3.2.9 Apoptosis analysis

Cleaved caspase 3 immunostaining was performed on WDs from control and flutamide exposed fetuses using standard methods, as detailed in section 2.6.1, to stain for apoptotic cells. Very few cleaved caspase 3 positive cells were detected, therefore a detailed stereological analysis was not appropriate and all positive cells were manually counted in each WD using an Axiolab microscope (Carl Zeiss Ltd, Hertfordshire, UK).

3.3 Results

3.3.1 Normal fetal Wolffian duct development

Figure 3.3 highlights the positions of the various reproductive organs within an e19.5 male (A) and female (B) fetal rat reproductive tract. In the male (A), the Wolffian duct develops lateral to the testis, connected proximally to the testis via the efferent ducts, while the distal segment of the WD, the future seminal vesicles (A), connects to the prostate. In the female (B), the MD connects proximally to the ovary, while the distal end connects to the urogenital sinus. At this age the female WD has fully regressed so cannot be seen, however, prior to regression, it would lie medial to the MD.

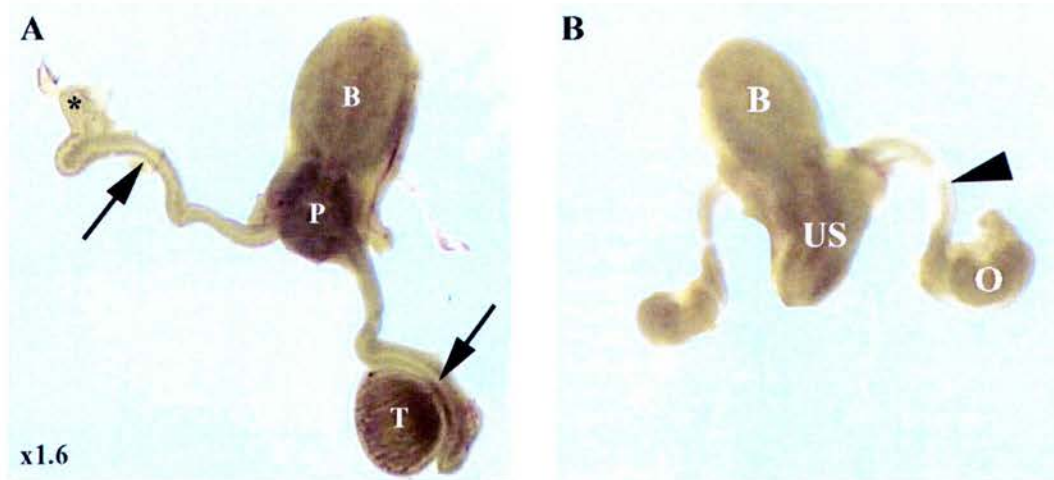


Figure 3.3 Representative reproductive tracts from e19.5 male (A) and female (B) rats. Note the male WD (arrow) connects proximally to the testis (T) via the efferent ducts (*) and distally inserts into the prostatic complex (P), situated above the bladder (B). In the female, the MD (arrowhead) leaves the ovary (O) and inserts into the urogenital sinus/bladder complex (US/B).

3.3.1.1 The timing and patterning of normal Wolffian duct development

Figure 3.4 shows images of male WDs isolated at e16.5 - e21.5. At e16.5, a remnant of the regressing MD can still be identified lying lateral to the WD. The MD has completely regressed by e17.5. The male WD remains a simple straight duct until e19.5, with coiling first evident at the caput (adjacent to the efferent ducts) and caudal ends of the future epididymal portion of the WD at e20.5 while the corpus remains relatively uncoiled. By e21.5, the full length of the epididymal portion of the WD appears highly convoluted, including the corpus. Distal to the future epididymal segment, the future vas deferens remains a straight duct throughout life.

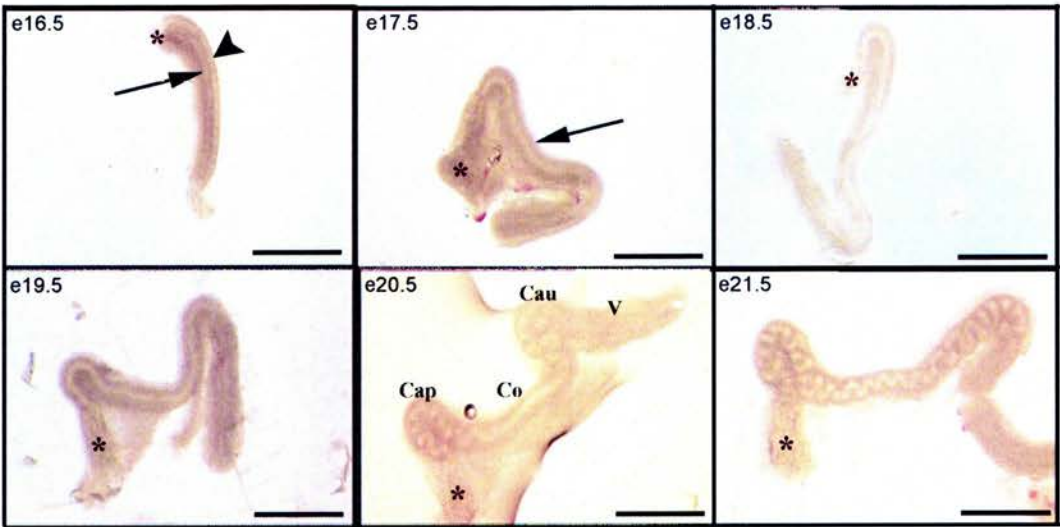


Figure 3.4 Representative WDs recovered from e16.5-e21.5 control fetuses showing an age-dependent increase in coiling/elongation. The remnant of the male MD is still visible in the male at e16.5 (arrowhead), medial to the WD (arrow). Note the appearance of coiling in the WD caput (Cap) and cauda (Cau) at e20.5 and along the corpus (Co) by e21.5. The efferent ducts (*) lie at the end proximal to the testis while the vas deferens (V) continues distally towards the prostate. Scale bar = 100 μ m. All images are at the same magnification.

This age dependent increase in coiling and development was confirmed quantitatively, by the demonstration of an increase in luminal length (Fig. 3.5). No significant increase ($p>0.05$) in WD luminal length was seen between e17.5-e18.5 or e18.5-e19.5, but a highly significant increase ($p<0.001$) was noted on consecutive days thereafter.

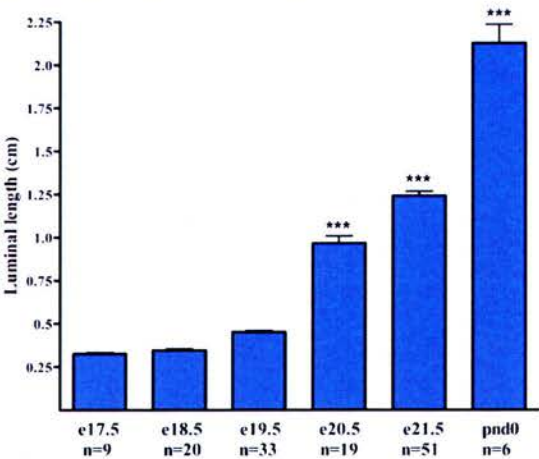


Figure 3.5 Age-dependent increase in coiling/elongation of the Wolffian duct (WD) between e17.5 and pnd0. The graph illustrates quantification of coiling (= luminal length) of the WDs. Note the progressive increase in luminal length of WDs from controls between e17.5 and pnd0. Values are mean \pm S.E.M. *** $p<0.001$, in comparison to respective value for preceding day.

3.3.1.2 Normal Wolffian duct histology

The WD is made up of 2 cellular compartments (Fig. 3.6). Simple columnar epithelial cells line the lumen of the duct with mesenchymal cells surrounding this epithelium: this histological organisation did not vary along the length of the WD or with age. The mesenchymal cells can be easily separated into two distinct populations: those directly surrounding the epithelial cells which are more densely packed, and termed here as inner stroma, and those that make up the rest of the “body” of the WD, referred to here as outer stoma. Note that at e17.5, the remnant of the regressing MD can still be identified.

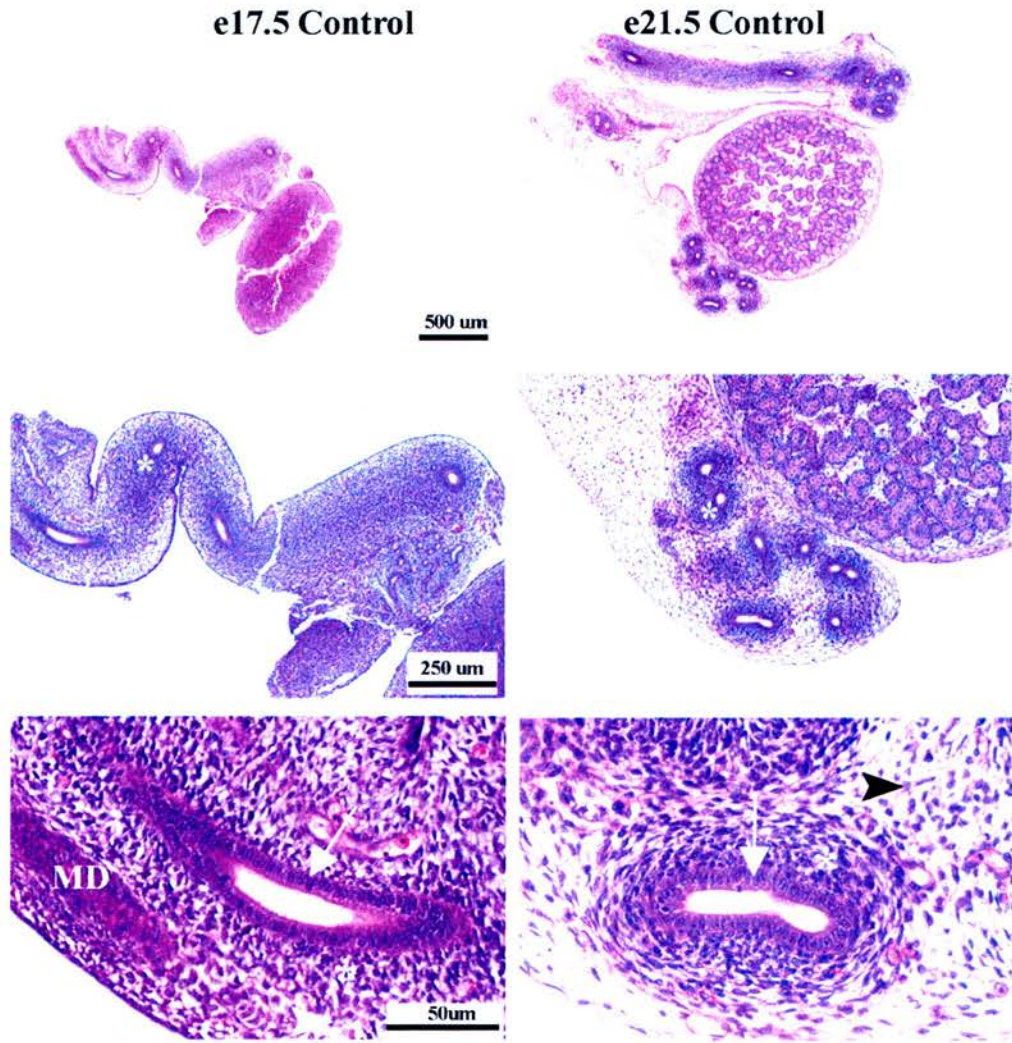


Figure 3.6 Demarcation of a e17.5 and e21.5 Wolffian duct (WD) stroma into the inner (IS; *) and outer stromal compartments (OS; arrowhead). Note that the IS (*) can be identified as the more dense compartment immediately adjacent to the epithelium (arrow). Note that the MD (MD) remnant can still be seen lateral to the WD at e17.5.

3.3.2 Normal morphology of adult male reproductive tract

Figure 3.7 shows a normal adult male reproductive tract (pnd 70), highlighting the position of the WD-derived tissues. The epididymis attaches proximally to the testis via the efferent ducts then connects distally to the vas deferens to carry sperm out of the body. The vas deferens remains a simple straight duct throughout life whereas the epididymis, in which coiling was initiated in the fetus, becomes even more convoluted and highly coiled during postnatal development.

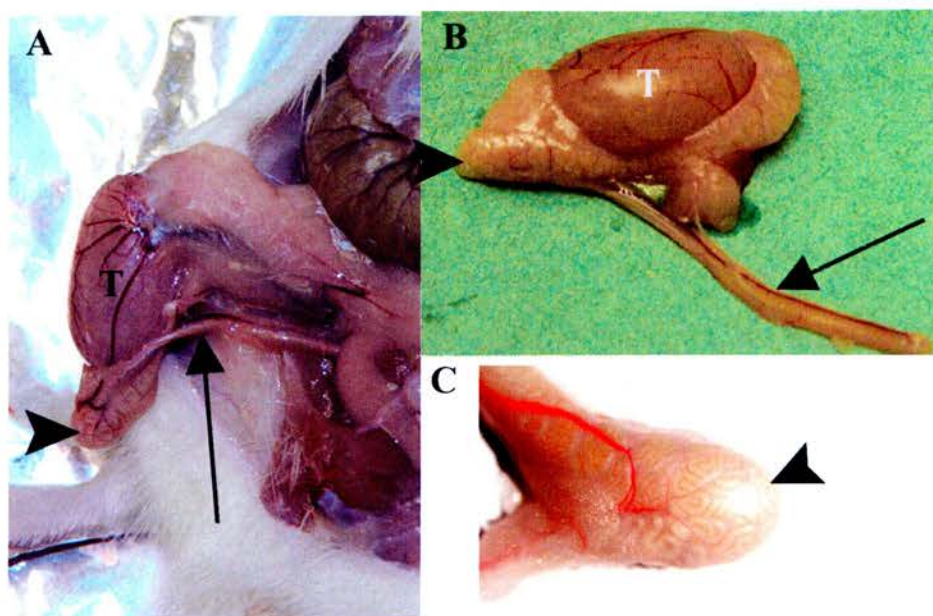


Figure 3.7 Representative male reproductive tract from a young adult rat (pnd70). Note that the epididymis (arrowhead) lies alongside the testis (T) then connects distally to the vas deferens (arrow). Panel C highlights the extensive coiling in the epididymis (arrowhead).

3.3.3 Effects of maternal flutamide exposure

3.3.3.1 Non-reproductive effects of flutamide

Consistent with previous studies (McIntyre *et al*, 2001; Foster and Harris, 2005), exposure to flutamide (50 or 100 mgkg⁻¹) did not result in any toxicological effects to the dam with no obvious problems with labour, no increase in fetal death (data not shown) or decrease in litter number ($p=0.38$). However, there was a slight decrease in maternal weight gain (Fig. 3.8) by the end of gestation (e21.5) between control dams and flutamide exposed dams (100 mgkg⁻¹) but this was not significant ($p=0.4$). At e21.5, there was no change in the bodyweight of female fetuses from flutamide-exposed mothers (50 or 100 mgkg⁻¹) compared to age matched controls (Fig 3.9). However, there was a significant decrease ($p<0.05$) in

male bodyweight at e21.5 between control fetuses and those from litters maternally exposed to high doses of flutamide (100 mgkg^{-1}). This was not evident in fetuses from dams exposed to low dose flutamide (Fig 3.9).

Bodyweight remained slightly reduced at pnd17 in males from litters exposed to high dose maternal flutamide (100 mgkg^{-1} , Fig 3.10) compared to age matched control males.

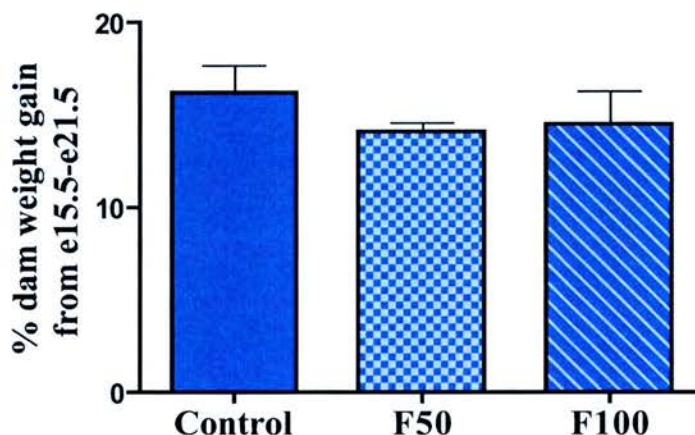


Figure 3.8 Maternal % weight gain at e21.5, relative to bodyweight at e15.5. Note the trend for reduced weight gain by e21.5 in dams exposed to flutamide (50 mgkg^{-1} , chequered bars; 100 mgkg^{-1} , striped bars) during pregnancy, but this was not significant. Values are means \pm S.E.M for 4-8 animals per group.

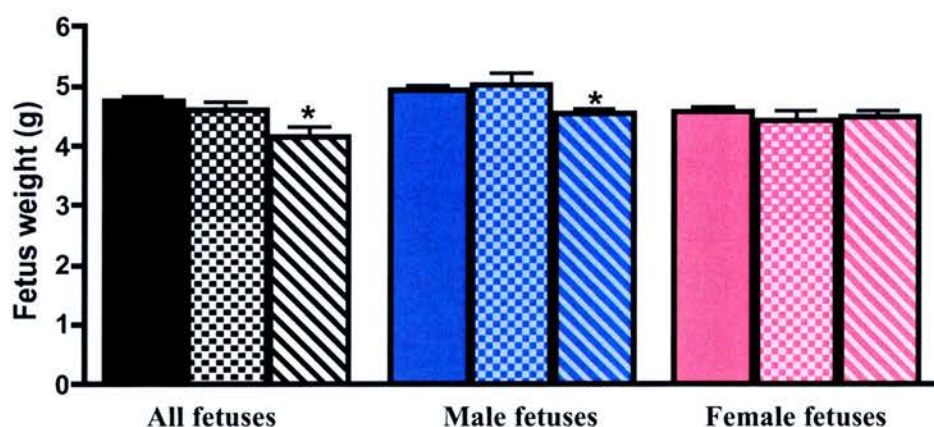


Figure 3.9 Bodyweight at e21.5 in fetuses from control (solid bars), low dose flutamide (50 mgkg^{-1} ; chequered bars) and high dose flutamide (100 mgkg^{-1} ; striped bars) litters. Note the decrease in fetal bodyweight from dams exposed to high dose flutamide (* $p < 0.05$) compared to age matched controls. This is likely to be due to the reduced bodyweight of male fetuses (* $p < 0.05$) from these litters as there was no change in female fetal bodyweight. Values are means \pm S.E.M for 9-30 animals per group.

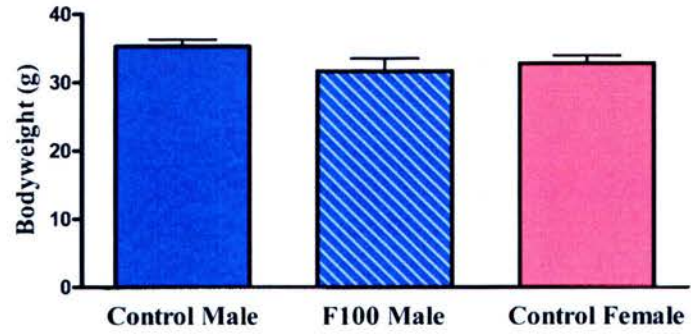


Figure 3.10 Bodyweight of *pnd17* animals from control litters and litters exposed to maternal flutamide treatment during gestation (100 mgkg^{-1}). Bodyweight remains reduced in *pnd17* males exposed to flutamide (100 mgkg^{-1} ; striped bar) during gestation compared to control males (blue bar), but this was less obvious than in fetal rats and was not significant. Values are means \pm S.E.M for 14-16 animals.

3.3.3.2 Effects of flutamide on the reproductive tract

Maternal exposure to either dose of flutamide (50 or 100 mgkg^{-1}), as expected, had no obvious effect on fetal female reproductive tract morphology but prevented normal masculinisation of fetal males. Normally, fetal sex can be determined externally by the distance between external genitalia and the anus (anogenital distance; AGD), with AGD significantly larger in fetal males than in females (Fig 3.11, $p<0.001$). However, maternal exposure to flutamide prevented normal masculinisation of fetal external genitalia, with AGD significantly reduced ($p<0.001$) in e21.5 males from flutamide-exposed litters to a length similar to that of control females. Exposure to flutamide (50 or 100 mgkg^{-1}) did not result in any change in female AGD at e21.5 ($p>0.05$).

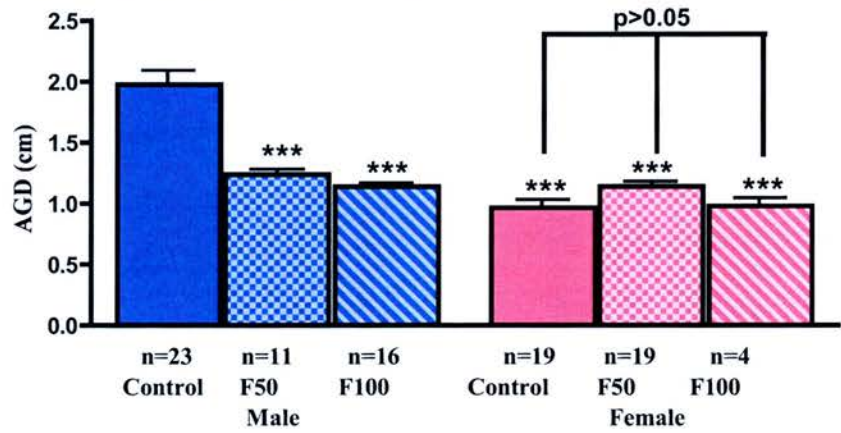


Figure 3.11 Anogenital distance (AGD) in e21.5 male (blue bars) and female (pink bars) fetuses from control (solid bars), low dose maternal flutamide (50 mgkg^{-1} ; chequered bars) and high dose maternal flutamide (100 mgkg^{-1} ; striped bars) litters. Note that AGD was significantly smaller in control and flutamide-exposed female fetuses (pink bars) than in males (solid blue bar) AGD in male flutamide-exposed fetuses (50 mgkg^{-1} , blue chequered

bars; 100 mgkg⁻¹, blue striped bar) was reduced compared to control males and was comparable to female AGD. Maternal exposure to either dose of flutamide did not alter female AGD (pink bars, $p>0.05$). *** $p<0.001$ compared to control male AGD. Values are means \pm S.E.M.

As shown in Fig 3.12, AGD remained reduced to female values ($p<0.001$) in pnd17 males from flutamide-exposed litters (100 mgkg⁻¹) compared to age matched controls.

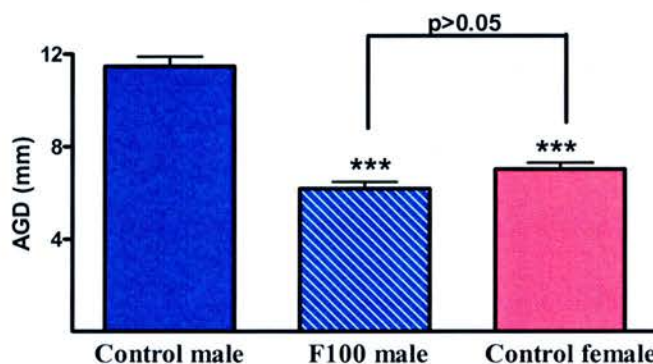


Figure 3.12 Anogenital distance (AGD) in pnd17 animals. Note the reduced AGD in males from litters exposed to flutamide in utero (striped bar; 100 mgkg⁻¹) compared to control males (blue bar): AGD in flutamide-exposed males was reduced to a level comparable with control female AGD (pink bar) ($p>0.05$). Values are means \pm S.E.M for 6-14 animals. *** $p<0.001$, compared to control male AGD.

Maternal exposure to flutamide prevented normal prostate formation in males and resulted in the presence of a vagina (data not shown). Testis descent was unaffected at e21.5 by maternal flutamide exposure (data not shown). Consistent with previous reports (Mylchreest *et al*, 1999; McIntyre *et al*, 2001; Foster and Harris, 2005), these abnormalities were still evident postnatally (data not shown).

3.3.4 Gross effects of flutamide on the fetal Wolffian duct and its adult derivatives

Maternal exposure to either dose of flutamide (50 or 100 mgkg⁻¹) had detrimental effects on the fetal WD, which persisted in the adult WD-derived tissues. These are detailed below.

3.3.4.1 Flutamide induced abnormalities in the fetal Wolffian duct

Maternal exposure to flutamide (50 or 100 mgkg⁻¹) impaired normal WD development but considerable variation was seen in the degree and phenotype of impairment between different ages, different doses of flutamide and even between littermates.

3.3.4.1.1 Effects of maternal treatment with flutamide on fetal Wolffian duct coiling and differentiation

Maternal exposure to flutamide interfered with the pattern and degree of WD coiling/development, as seen in Fig. 3.13. Consistent with previous studies (Bowman *et al*, 2003) that have interfered with androgen action *in utero*, considerable variation was noted between animals in the degree of WD coiling after maternal exposure to flutamide: this was more apparent at later fetal ages (e20.5 - 21.5) when WDs are more differentiated in fetuses from control mothers. To take account of this variability, WDs were analysed from 6-51 animals from at least 3 litters per age/treatment group.

An age-dependent increase was noted in the degree of coiling and hence differentiation of WDs from flutamide exposed fetuses, but this was reduced compared to age matched controls (Fig. 3.13). As in controls, no coiling was seen in WDs from flutamide-exposed animals (50 or 100 mgkg⁻¹) at e18.5 or e19.5 (Fig. 3.13). WD coiling was dramatically reduced in all e20.5 and e21.5 fetuses from flutamide exposed mothers (50 or 100 mgkg⁻¹, Fig. 3.13), with some WDs less coiled than others (data not shown). In fetuses from low dose flutamide exposed dams (50 mgkg⁻¹), the onset of WD morphological differentiation was not altered, with coiling first evident at e20.5, as in control fetuses (Fig. 3.13). However, the onset of WD coiling varied in fetuses from dams exposed to high dose flutamide (100 mgkg⁻¹) with coiling evident in some WDs by e20.5 while some WDs still showed no sign of coiling by e21.5 (Fig. 3.13). In control WDs, coiling was first evident at the caput and cauda, with coiling not evident in the corpus until a later age (Fig. 3.13). However, the patterning of WD coiling was altered by maternal exposure to flutamide (50 mgkg⁻¹) with coiling first evident in the caput and the proximal end of the corpus at e20.5 and appearing to progress along the duct towards the cauda by e21.5 (Fig. 3.13). It was not easy to distinguish the pattern of WD coiling in fetuses from dams exposed to high dose flutamide (100 mgkg⁻¹) since coiling was still not apparent in many of these ducts at e21.5.

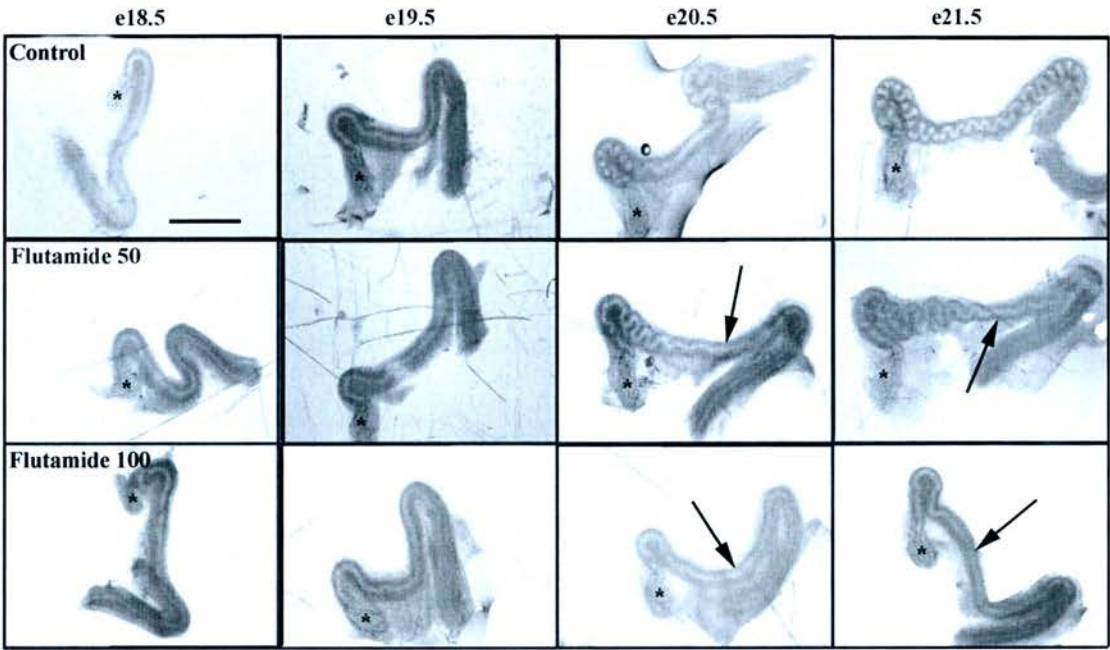


Figure 3.13 Age-dependent increase in coiling/elongation of the Wolffian duct (WD) between e18.5 and e21.5. Representative WDs recovered from control fetuses (top row) and fetuses exposed to 50 mgkg⁻¹ flutamide (middle row) or 100 mgkg⁻¹ flutamide (bottom row) at e18.5 (first column), e19.5 (second column), e20.5 (third column) and e21.5 (final column) are illustrated. Note the reduced coiling in WDs from flutamide-exposed animals at e20.5 and e21.5 (arrow) when compared to the age matched control (top row). Scale bar = 100 μ m. All images are at the same magnification. * - efferent ducts

These qualitative observations were quantified by measuring the length of the WD lumen. At e17.5-e19.5, no difference was noted in the luminal length of WDs from fetuses from dams exposed to flutamide (50 mgkg⁻¹ or 100 mgkg⁻¹). At e20.5 and e21.5, however, flutamide-exposed fetuses showed a highly significant reduction ($p<0.001$) in WD coiling and luminal length, compared to age matched controls (Fig. 3.14). At e21.5, coiling was more dramatically reduced in fetuses from dams exposed to 100 mgkg⁻¹ flutamide than those exposed to 50 mgkg⁻¹ (Fig. 3.14). This dose dependent difference was highly significant ($p<0.001$).

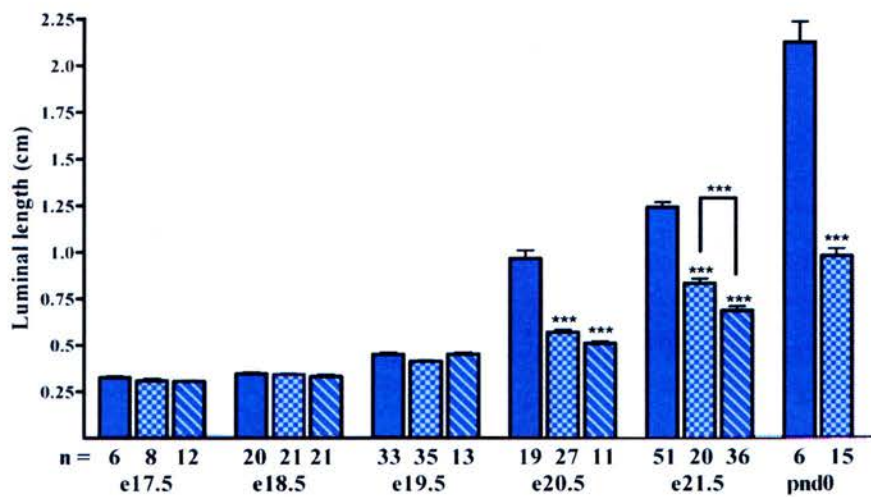


Figure 3.14 Quantification of coiling (= luminal length) of e17.5 – pnd0 WDs from control and flutamide-exposed litters. Note the progressive increase in luminal length of WDs from controls between e18.5 and pnd0 (solid blue bars). There was a significant reduction in luminal length at e20.5 and e21.5 in WDs from animals exposed in utero to either 50 mgkg⁻¹ (chequered bars) or 100 mgkg⁻¹ (striped bars) flutamide, when compared to controls, whereas at e18.5 and e19.5, no difference was evident. Values are mean ± S.E.M, ***p<0.001, in comparison to the respective control value.

3.3.4.1.2 Maternal flutamide treatment does not alter fetal Wolffian duct width

As evident from Fig. 3.15, there was no significant change in the overall width of the WD in fetuses from dams exposed to flutamide (50 mgkg⁻¹), compared to age matched controls. This was not measured in WDs from fetuses from dams exposed to 100 mgkg⁻¹ flutamide.

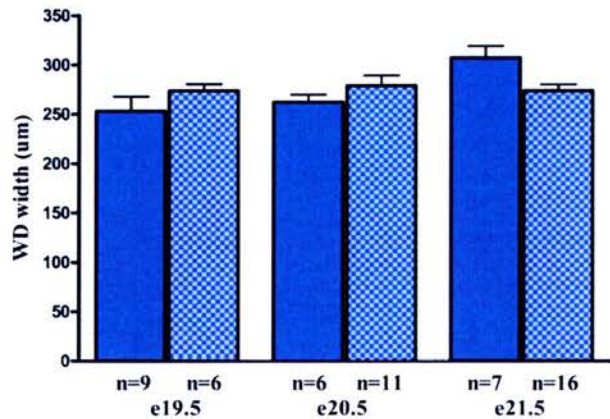


Figure 3.15 Width of control and flutamide exposed Wolffian ducts at e19.5-e21.5. WD width did not change significantly with age or in fetuses from dams exposed to flutamide during pregnancy (50 mgkg⁻¹, chequered bars), compared to age matched controls (solid blue bars). Values are means ± S.E.M.

3.3.4.1.3 Flutamide-induced loss of Wolffian duct segments

As well as the reduction in coiling mentioned in section 3.4.4.1.1, some WDs from flutamide exposed fetuses appeared incomplete at e21.5 with thinning of the epithelium and missing corpus/cauda segments (Fig. 3.16): this was never seen in control WDs and affected WDs always had a short lumen apparent at the caput. This phenotype of missing segments was first noted at e21.5 and was similar in low and high dose flutamide litters, however, there was an increased prevalence in fetuses from high dose flutamide litters in which 11% of WDs recovered from animals exposed to flutamide had incomplete lumens compared to a 5% prevalence in fetuses from low dose flutamide litters (Table 3.1). This was not seen in WDs from flutamide-exposed animals prior to e20.5. This is in contrast to the natural regression of WDs in females, in which by e18.5 the WD has fully regressed and is no longer visible (chapter 6).

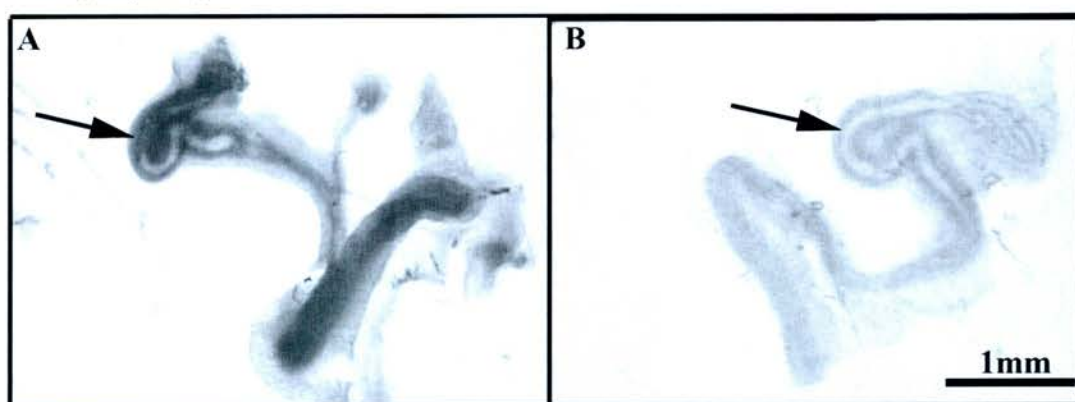


Figure 3.16 *Flutamide-induced interruption to the lumen of the WD at e21.5 in occasional animals. Note that exposure to both low (50 mgkg^{-1} , A) and high (100 mgkg^{-1} , B) dose maternal flutamide resulted in lumens only being apparent at the caput end of the WD (arrow). Both images are at the same magnification.*

Table 3.1 *Number of incomplete WDs in control and flutamide-exposed animals.*

Age	Treatment	Number of incomplete WDs (%)	Number of complete Wds
E18.5	Control	0	10
	Flutamide 50 mgkg ⁻¹	0	21
	Flutamide 100 mgkg ⁻¹	0	21
E19.5 ¹	Control	0	37
	Flutamide 50 mgkg ⁻¹	0	47
	Flutamide 100 mgkg ⁻¹	0	35
E20.5	Control	0	25
	Flutamide 50 mgkg ⁻¹	0	68
	Flutamide 100 mgkg ⁻¹	2 (7%) ²	29
E21.5	Control	0	24
	Flutamide 50 mgkg ⁻¹	2 (5%) ²	39
	Flutamide 100 mgkg ⁻¹	4 (11%) ²	34
Pnd17	Control	0	16
	Flutamide 100 mgkg ⁻¹	1 (13%) ³	7
Pnd42	Control	0	12
	Flutamide 100 mgkg ⁻¹	5 (63%) ²	3
Adults	Control	0	18
	Flutamide 100 mgkg ⁻¹	10 (83%) ³	2

² _____
¹ At e19.5, the Wolffian duct in control female rats has completely regressed
² Note that these WDs were all from the same litter (e20.5 F100 – 1 of 3, e21.5 F50 - 1 of 6, e21.5 F100 - 1 of 3, and pnd17 F100 1 of 1)
³ Note that these reproductive tracts were found in males from 2 of 2 litters at pnd42 and 3 of 3 litters in adulthood.

3.3.4.2 Persistence of maternal flutamide-induced gross abnormalities in postnatal Wolffian duct-derived tissues

The abnormalities seen in the fetal WD not only persisted in the WD-derived tissue in postnatal life, but in the majority of postnatal animals abnormalities were more prevalent and more severe than in the fetus. Considerable variation was noted in the degree of abnormality seen in WD-derived adult tissues, including underdeveloped or incomplete epididymides, lack of vas deferentia, and underdeveloped or vestigial seminal vesicles (Fig. 3.17 and 3.18). As in the fetus, at pnd17 (i.e. before puberty) 13% of males from dams exposed to 100 mgkg⁻¹ flutamide had incomplete WD-derived tissues, with the corpus segment of the epididymis often absent: the prevalence of this phenotype rose to 63% by pnd42 (Table 3.1). Similarly, in adult males from dams exposed to 100 mgkg⁻¹ flutamide, 83% of epididymides and/or vasa deferentia studied were incomplete (Table 3.1). In five adult males, there was near complete absence of all WD-derived tissues.

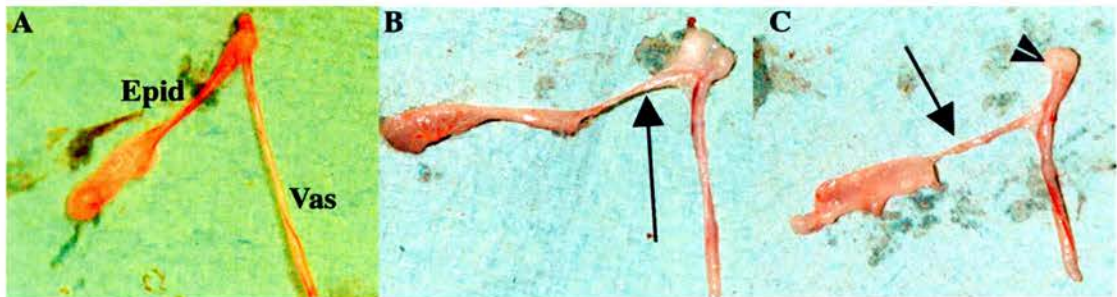


Figure 3.17 Wolffian duct-derived tissues in males from control (A) and maternal flutamide treated (100 mgkg⁻¹, B and C) animals at pnd42. Note the thinning of the corpus (arrow, B and C) and the vestigial caudal segment (arrowhead, C) in epididymides from males exposed to maternal flutamide during gestation, compared to age matched control males (A). All images are the same magnification.

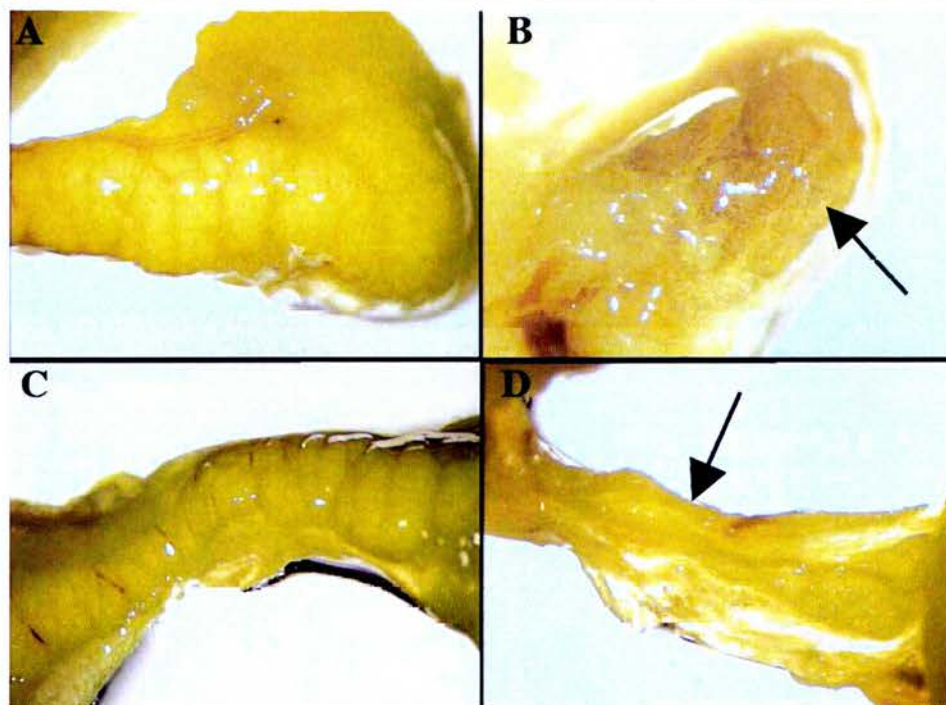


Figure 3.18 Adult (pnd70) epididymides from control males (A and C) and males from litters exposed to maternal flutamide treatment (B and D, 100 mgkg⁻¹). Note the abnormal caput from the flutamide-exposed animal (arrow, B) compared to the highly convoluted caput from the control epididymis (A). Furthermore, note the absence of any obvious corpus in the flutamide male (arrow, D) compared to the normal coiled corpus (C). All images are at the same magnification.

3.3.5 Flutamide-induced histological abnormalities in fetal WDs

Maternal exposure to flutamide (50 or 100 mgkg⁻¹) did not alter the cellular compartmentalisation of the fetal WD into epithelial and stromal compartments, as described in section 3.3.1.2 (Fig. 3.19).

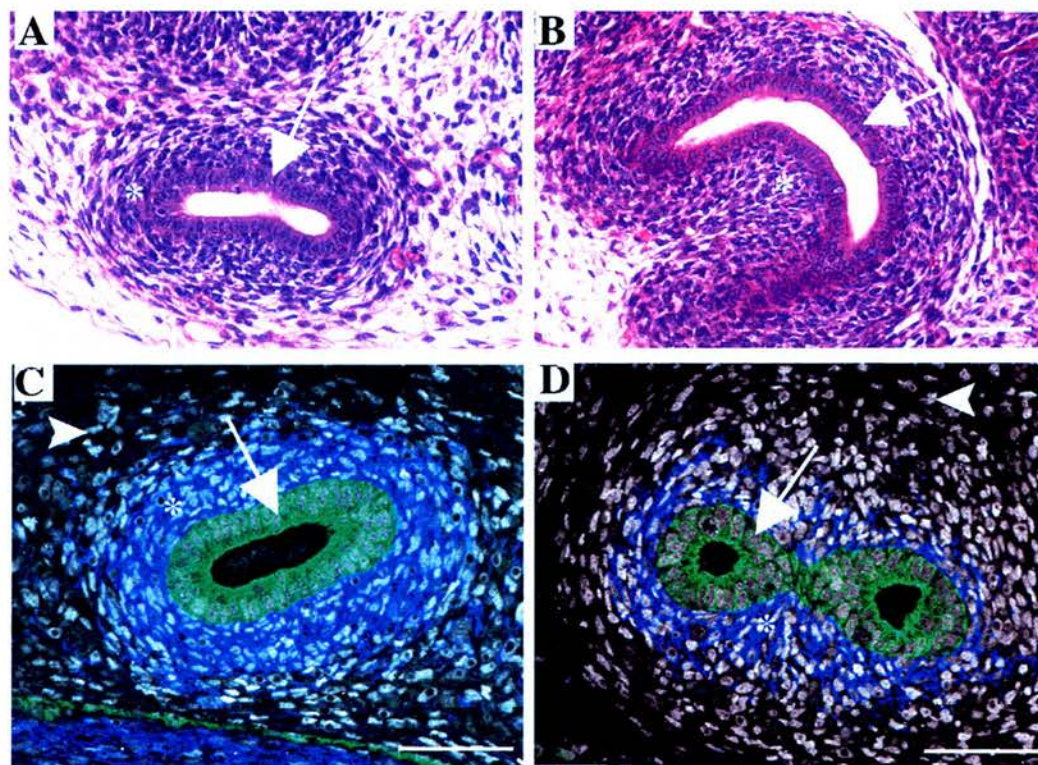


Figure 3.19 Cellular compartments in e21.5 Wolffian ducts from control (A and C) and flutamide-exposed (B and D) males. Note that flutamide exposure did not alter the compartmentalisation of the WD into the epithelium (arrow) and the surrounding stromal compartments. Note also that the stroma can easily be distinguished into the more densely compacted inner stroma (*; in panels C and D, immunostained blue for smooth muscle actin), immediately proximal to the epithelium (arrow; in panels C and D; immunostained green for cytokeratin), and the outer stroma (arrowhead; in panels C and D, SMA negative, nuclei are counterstained with DAPI, shown as white). Scale bars = 50 μm

3.3.5.1 Gross changes to the epithelial compartment after flutamide exposure

Examination of WDs revealed no obvious histological differences between WDs from control and flutamide-exposed fetuses at e18.5 or e19.5 (data not shown). However, as seen in Fig. 3.20, histological abnormalities became apparent by e21.5 in WDs from fetuses exposed to flutamide (50 or 100 mgkg^{-1}), including loss of epithelium lining the lumen, widening or narrowing of the lumen and flattening of the epithelium compared to that of controls, sometimes even appearing fibroblast-like (Fig. 3.20). The extent of this flattening varied between WDs but was not seen in controls or flutamide-exposed WDs at any earlier age.

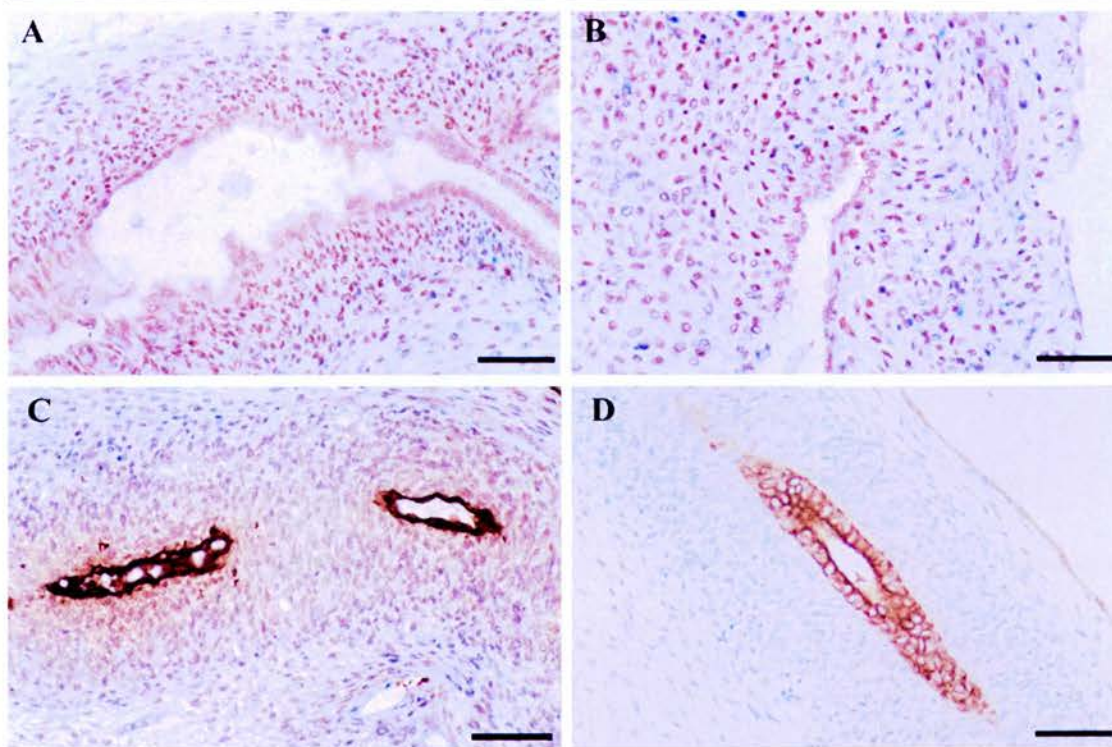


Figure 3.20 Representative abnormalities in epithelia of e21.5 WDs from low (50 mgkg^{-1} , A & B, stained for a nuclear receptor) and high (100 mgkg^{-1} , C & D, stained for cytokeratin) dose flutamide litters. Note the widened lumen seen in some WDs (A) compared to the flattened lumen in others (C). There is an apparent loss of epithelium lining the lumen in several WDs (A-C) while in other WDs, the epithelium was still present but the cells were reduced in height (D). Scale bars = $50 \mu\text{m}$

3.3.5.2 Quantification of epithelial cell abnormalities after flutamide exposure

As noted above, maternal treatment with flutamide induced abnormalities in the epithelial compartment of the WD. The apparent reduction in epithelial cell height can be seen in Fig. 3.21 and was confirmed quantitatively at e18.5 and e21.5 (Fig. 3.22). At e18.5, the reduction in WD epithelial cell height was not as obvious microscopically as in e21.5 WDs; however, when quantified, there was a trend for reduced epithelial cell height in WDs from e18.5 fetuses of dams exposed to flutamide compared to age matched controls but this was not significant. By e21.5, maternal flutamide exposure significantly reduced fetal WD epithelial cell height ($p < 0.01$). This reduction was more dramatic in the corpus and cauda portions of the WD and was most pronounced in fetuses from dams exposed to 100 mgkg^{-1} flutamide. No significant difference was noted in the width of WD epithelial cells in flutamide-exposed animals (100 mgkg^{-1}) compared to controls (Fig. 3.23).

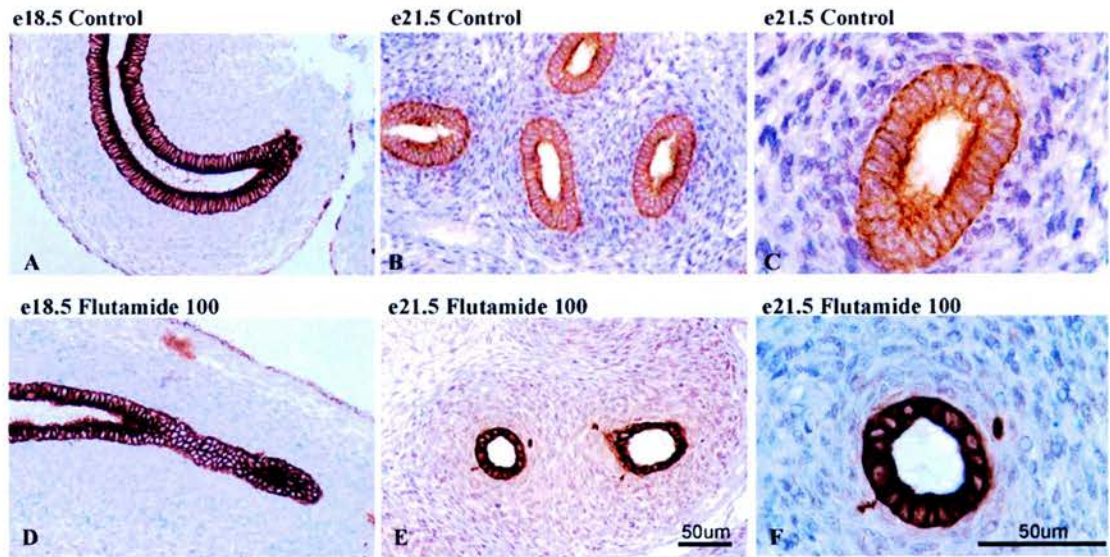


Figure 3.21 Reduced epithelial cell height at e18.5 and e21.5 in flutamide-exposed (100 mg/kg-1, D-F) WDs, compared to age matched controls (A-C). Note that the reduced epithelial cell height (immunostained for cytokeratin) in WDs in the flutamide-exposed animal is not as apparent at e18.5 (A compared to D) but is obvious at e21.5 (compare B to E and C to F). Images are all from the caput region of a WD, the segment of the WD where epithelial height is least reduced by flutamide exposure. Scale bars = 50µm

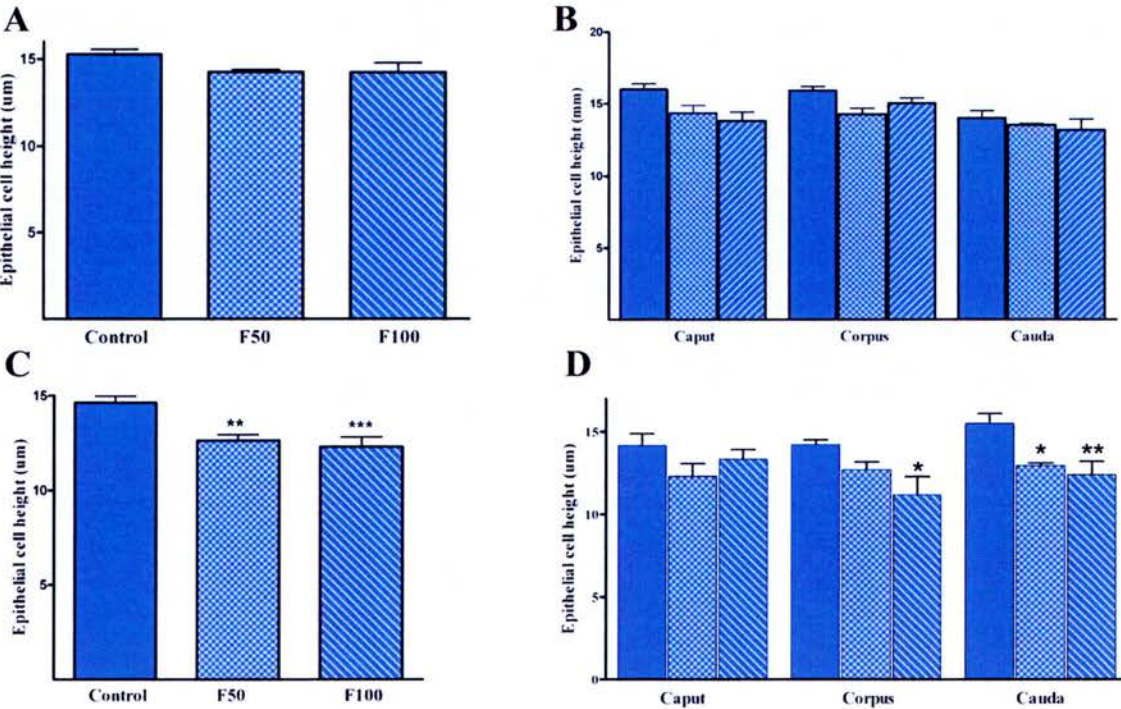


Figure 3.22 Quantification of epithelial cell height at e18.5 (A & B) and e21.5 (C & D) in Wolffian ducts (WDs) from controls (solid blue bars) and animals exposed to 50 mg/kg⁻¹ (chequered bars) or 100 mg/kg⁻¹ (striped bars) flutamide. At e18.5, there was a trend for reduced epithelial cell height but this was not significant ($p>0.05$, A & B). Maternal flutamide

exposure resulted in a highly significant reduction in epithelial cell height at e21.5 in the WD as a whole (C; 50 mgkg⁻¹ $p < 0.01$, 100 mgkg⁻¹ $p < 0.001$). However, when each segment of the epididymal portion of the WD was analysed individually, epithelial cell height was found to be reduced more in the corpus and cauda portions of the future epididymis after flutamide treatment (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, in comparison to respective control value. Values are means \pm S.E.M for 5-7 animals per treatment, from at least 3 different litters.

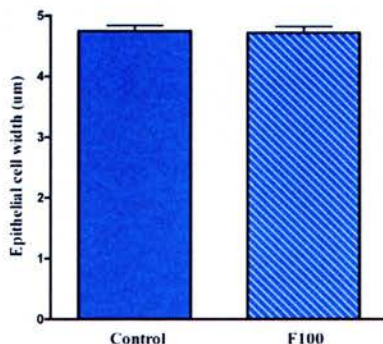


Figure 3.23 Epithelial cell width at e21.5 in Wolffian ducts (WDs) from controls (solid blue bars) and animals exposed to 100 mgkg⁻¹ (striped bars) flutamide. Note that no difference was seen in WD epithelial cell width between control and flutamide-exposed litters. Values are means \pm S.E.M for 3 animals per group.

3.3.6 Prevalence of apoptotic cells in fetal Wolffian ducts

Apoptotic cells were rarely detected (typically 1 cell per section) in either epithelial or stromal compartments of WDs from either control or flutamide exposed fetuses at any age examined (Fig. 3.24).

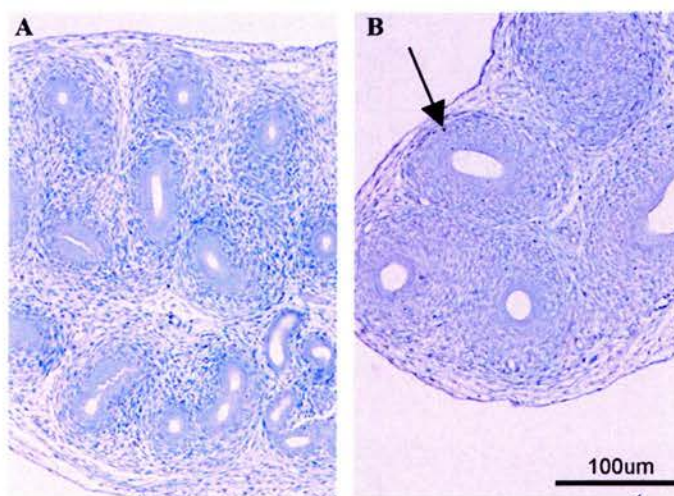


Figure 3.24 Frequency of apoptotic cells (immunopositive for cleaved caspase 3; black staining) in representative e21.5 Wolffian ducts (WDs) from a control (A) and an animal exposed in utero to 100 mgkg⁻¹ flutamide (B). Note that few apoptotic cells were seen in either control or flutamide-exposed WDs (arrow). Scale bar = 100 um.

3.3.7 Analysis of cell proliferation in fetal Wolffian ducts

Cell mitosis was evident in all compartments of WDs from control and flutamide-exposed animals at all ages (Fig. 3.25). Immunostaining for phospho-histone H3 suggested fewer mitotic cells were present in WDs from flutamide-exposed fetuses compared to controls (Fig. 3.25). This was confirmed quantitatively highlighting a reduction in the number of mitotic epithelial and stromal cells per WD, compared to age-matched controls, at all ages studied (Fig. 3.26-3.28). Note that a significant reduction in the number of mitotic cells was apparent in the stroma at e19.5 and e20.5 (Fig. 3.26 & 3.27) but was not seen in the epithelium until e21.5 (Fig. 3.28). Furthermore, the reduction in number of mitotic cells became more apparent at e21.5 and maternal exposure to 100 mgkg⁻¹ flutamide resulted in a greater reduction in the number of mitotic cells than did 50 mgkg⁻¹ (Fig. 3.28).

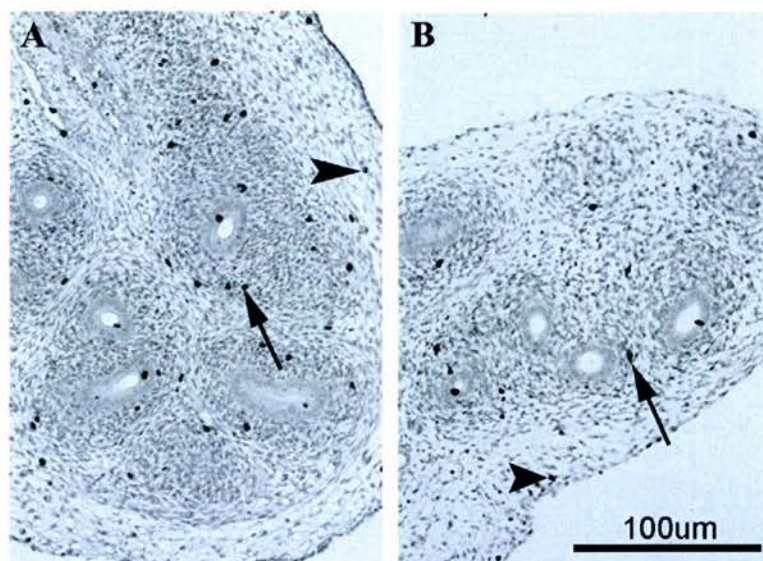


Figure 3.25 Frequency of mitotic cells (immunopositive for phospho-histone H3; black staining) in representative e21.5 Wolffian ducts (WDs) from a control (A) and an animal exposed in utero to 50 mgkg⁻¹ flutamide (B). Note the numerous mitotic cells in the inner stroma (arrows), outer stroma (arrowhead) and epithelium. Scale bar = 100 μ m

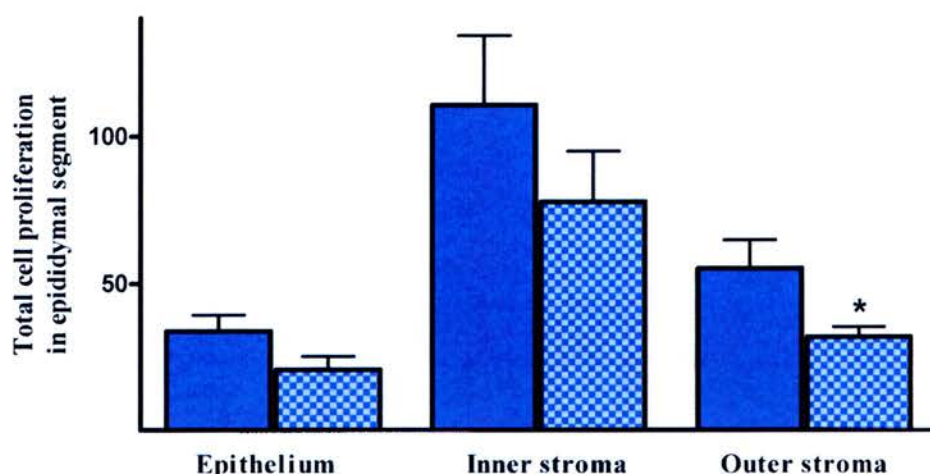


Figure 3.26 Total number of mitotic epithelial and stromal cells in the entire epididymal segment of the WD at e19.5 in controls (solid blue bars) and animals exposed to 50 mgkg⁻¹ (chequered bars) flutamide. Note the reduction in number of mitotic cells in WDs from animals exposed to flutamide compared to controls: this trend was apparent in all cellular compartments but was only significant in the outer stromal compartment at this age. * $p < 0.05$, in comparison with respective control values. Values are means \pm S.E.M for 8-12 animals.

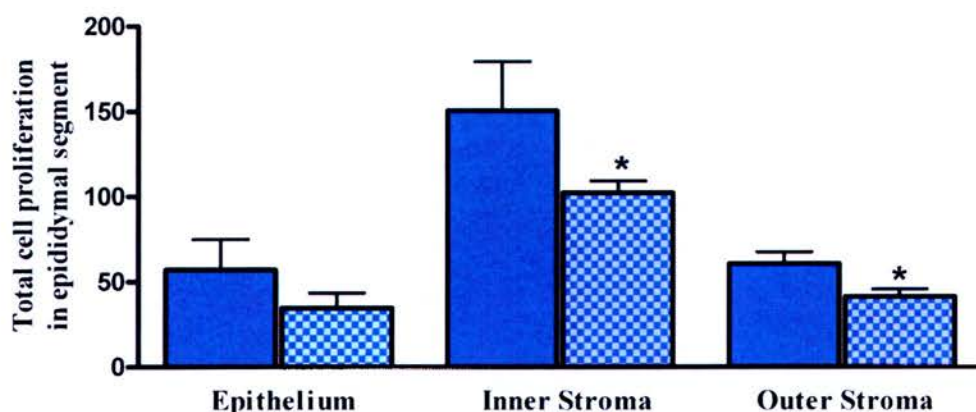


Figure 3.27 Total number of mitotic epithelial and stromal cells in the entire epididymal segment of the WD at e20.5 in controls (solid blue bars) and animals exposed to 50 mgkg⁻¹ (chequered bars) flutamide. Note the reduction in number of mitotic cells in WDs from animals exposed to flutamide compared to controls: this trend was apparent in all cellular compartments but was only significant in the stromal compartment at this age. * $p < 0.05$, in comparison with respective control value. Values are means \pm S.E.M for 8-11 animals.

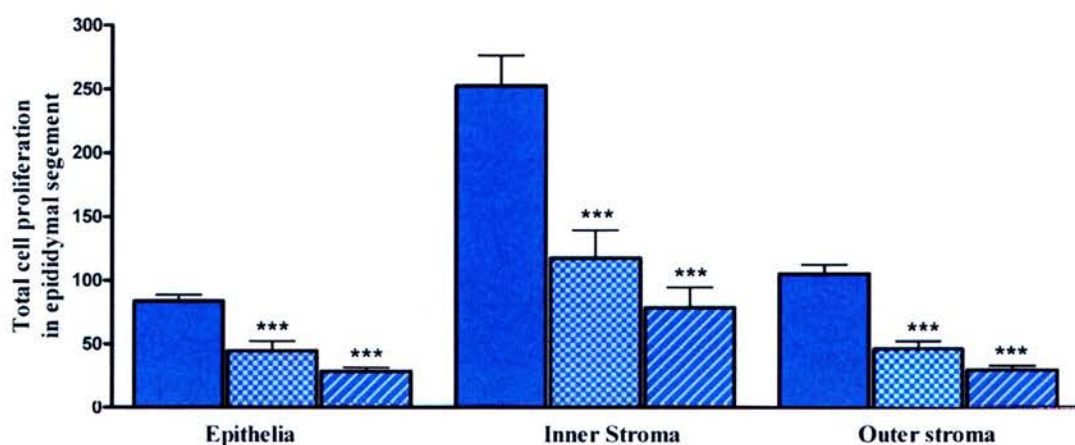


Figure 3.28 Total number of mitotic epithelial and stromal cells in the entire epididymal segment of the WD at e21.5 in controls (solid blue bars) and animals exposed to 50 mgkg⁻¹ (chequered bars) or 100 mgkg⁻¹ (striped bars) flutamide. Note the reduction in number of mitotic cells in WDs from animals exposed to flutamide compared to controls: this was apparent in all cellular compartments and was more dramatic in WDs from high dose flutamide litters compared to those from low dose litters. *** $p < 0.001$, in comparison to respective control value. Values are means \pm S.E.M for 12-16 animals.

Cell proliferation data was expressed as the overall number of mitotic cells in the complete epididymal portion of the WD rather than per 100 μ m epithelium, so as to take into account the treatment-induced reduction in WD length. If this correction was not applied, a similar treatment-induced reduction in cell mitosis was evident but was of smaller magnitude.

3.3.7.1 Epithelial mitotic index

Analysis of the epithelial mitotic index, at e21.5, yielded comparable results as the method described in section 3.2.1.2, with epithelial mitosis reduced ($p < 0.05$) in flutamide-exposed WDs, compared to control WDs (Fig. 3.29). This suggests that the analytical method used above reflects the mitotic index.

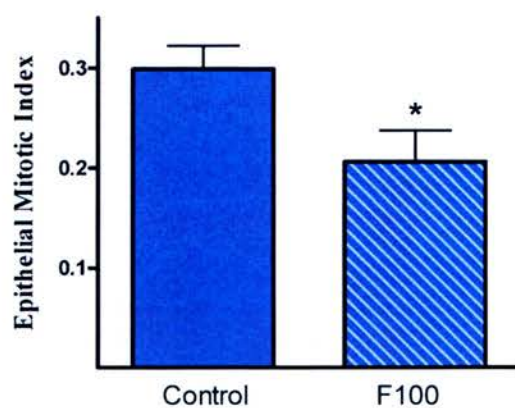


Figure 3.29 Epithelial mitotic index in the entire epididymal segment of the WD at e21.5 in controls (solid blue bars) and animals exposed to 100 mgkg^{-1} (striped bars) flutamide. Note the flutamide-induced reduction in mitotic index in the epithelial compartment at e21.5 compared to age-matched controls. * $p < 0.05$, in comparison to respective control value. Values are means \pm S.E.M for 3 animals

3.4 Discussion

In males, the WD differentiates during neonatal life to form the epididymis, vas deferens and seminal vesicles (Wilson *et al*, 1981). In female rats, due to a lack of androgens the WD naturally regresses between e16.5 and e18.5 (see chapter 6) whereas at this age in males, androgen action is believed to stabilise the WD, allowing it to differentiate subsequently into its adult derivatives (George, 1994; Huhtaniemi, 1994). In our rat colony, the male MD has completely regressed by e17.5, leaving the simple straight WD lying alongside the testis. Morphological differentiation can be seen by coiling in the future epididymis of the fetal WD around e20.5 suggesting that the developmental window for WD morphological differentiation is between e19.5 and birth: this coincides with the peak in testicular testosterone production in male rats at e19.5 (Warren *et al*, 1972). These findings agree with previously published timings (Barlow and Foster, 2003). By e21.5, the WD can easily be distinguished into its adult derivative organs with the future epididymis already appearing highly coiled suggesting that the pattern of the reproductive tract is established, probably by androgens, during fetal life.

There are many anti-androgenic compounds available that interfere with androgen action in vivo using different modes of action, such as linuron, a weak AR antagonist (McIntyre *et al*, 2000), DBP, which reduces fetal testosterone production by the testis (Mylchreest *et al*, 1999), and flutamide, a competitive AR antagonist (Imperato-McGinley *et al*, 1992) (see section 1.5.2.2). Flutamide was selected for these studies as, unlike DBP, its mode of action has been well characterised as competition with testosterone or DHT for binding to the AR (Peets *et al*, 1974; Zuo *et al*, 2002; Schaufele *et al*, 2005). Furthermore, flutamide is believed to be a more potent AR antagonist than linuron: gestational exposure to linuron resulted in no epididymal abnormalities in male fetuses (McIntyre *et al*, 2002). Furthermore, gestational exposure to flutamide (100 mgkg⁻¹) resulted in a greater prevalence of epididymal abnormalities in the adult (100% epididymides absent/partially developed) than from exposure to DBP (500 mgkg⁻¹; 50% epididymides absent/partially developed) (Mylchreest *et al*, 1999).

The doses of flutamide selected for these studies were based on previous studies which also used flutamide to interrupt male reproductive development (Imperato-McGinley *et al*, 1992; Bentvelsen *et al*, 1995; Mylchreest *et al*, 1999; McIntyre *et al*, 2001; Hotchkiss *et al*, 2002). Dose response studies undertaken by Imperato-McGinley and colleagues highlighted that exposure to just 24 mgkg⁻¹ flutamide completely abolished prostate differentiation but had no obvious effect on WD development while exposure to 100 mgkg⁻¹ flutamide markedly

impaired WD differentiation with WD-derived structures often absent in the adult (Imperato-McGinley *et al*, 1992). These differential effects on different reproductive structures are likely to be due to the higher local concentrations of testosterone in the developing WD than in tissues derived from the urogenital sinus (Veyssiere *et al*, 1982). Doses above 100 mgkg⁻¹ flutamide (200 or 300 mgkg⁻¹) did not markedly increase the prevalence of these epididymal abnormalities (Imperato-McGinley *et al*, 1992) suggesting that maternal exposure to 100 mgkg⁻¹ flutamide is sufficient to induce a high prevalence of epididymal abnormalities. In this thesis, dams were also exposed to 50 mgkg⁻¹ flutamide in order to induce less severe abnormalities without inducing a complete loss of WD tissues therefore allowing investigation of the effects of partial androgen action inhibition and subsequent gene expression.

Consistent with previous studies (McIntyre *et al*, 2001; Foster and Harris, 2005), flutamide did not result in any obvious toxicological effects in the dams: for example, there were no signs of premature labour, no increased prevalence of fetal deaths and no obvious maternal health issues. Maternal exposure to flutamide, however, did decrease maternal weight gain. This is probably due to the decrease in male, but not female, fetal bodyweights suggesting that androgens may play a role in determining male fetal bodyweight. It is unclear whether this is due to flutamide disturbing androgen signalling in the mother or in the male fetus itself. Previous studies have highlighted a similar decrease in dam weight gain after treatment with 50 mgkg⁻¹ flutamide but without any significant change in pup weight (Mylchreest *et al*, 1999; McIntyre *et al*, 2001): currently there is no literature on pup weight after exposure to 100 mgkg⁻¹ flutamide. The reduction in bodyweight persisted in males postnatally, suggesting that postnatal bodyweight may be predetermined during fetal life. This is consistent with reports that exposure to glucocorticoids, especially during late gestation, alters offspring birthweight and the effect of this persists into postnatal life, even predicting adult morbidity (Seckl, 2004).

As previously reported maternal exposure to flutamide, at the doses used in this study, impaired androgen-dependent reproductive tract development in male offspring (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1999; McIntyre *et al*, 2001). As expected, female fetuses were unaffected by flutamide exposure thus confirming the accepted hypothesis that androgens play no role in female fetal development. However, male fetuses from dams exposed to either low (50 mgkg⁻¹) or high (100 mgkg⁻¹) doses of flutamide, had no prostate, reduced AGD and impaired WD development. These abnormalities persisted into adulthood confirming that the patterning and establishment of the reproductive tract is set up in the fetus and interfering with androgen action during this period permanently alters reproductive

tissues. This is consistent with previous studies (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1999; McIntyre *et al*, 2000; McIntyre *et al*, 2001) and confirms that we have established a model of impaired androgen action in our rat colony, comparable with other groups.

Previous studies have reported that flutamide exposure *in utero* results in abnormal epididymides in adults but these studies do not identify whether the WD formed and stabilised fetally but later degenerated during differentiation or whether it failed to form and/or stabilise (Mylchreest *et al*, 1999; McIntyre *et al*, 2001; Foster and Harris, 2005). In the present studies, WDs from flutamide-exposed males were present at e17.5 - e19.5 and appeared normal in all animals, with no obvious morphological differences or reduction in luminal length compared with age matched controls. This suggests that flutamide-exposed WDs initially form normally and are morphologically stabilised but it is unknown whether these WDs are functional at the biochemical level therefore this warrants further investigation. In the female, the WD regresses at least partially due to apoptosis suggesting that the WD is dying rather than simply not differentiating (discussed in chapter 6) (Dyche, 1979; Jirsova and Vernerova, 1993). In contrast, minimal cell apoptosis was detected in WDs from flutamide-exposed males at any age studied. Together these findings suggest that the doses of flutamide administered may not completely block the AR and so may not reduce androgen action sufficiently to impair WD stabilisation and cause it to degenerate, as occurs in the female (Jirsova and Vernerova, 1993). This difference could reflect the higher levels of testosterone reported to be available at e18.5, when the WD is undergoing stabilisation, than at e20.5, when the WD is morphologically differentiating thus making it more difficult for flutamide to block androgen action (Habert *et al*, 1992). Alternatively, WD stabilisation may require relatively low levels of androgens, less than that required for differentiation, or WD stabilisation and differentiation may be regulated by different mechanisms. This highlights WD development as a 2-phase process, each of which may be differentially controlled. It is likely that the former hypothesis is true since previous researchers have reported that it is easier to inhibit androgen dependent growth of immature sex accessory tissues than it is to initiate degeneration and apoptosis (Lambright *et al*, 2000). Insight from patients with complete androgen insensitivity syndrome (CAIS) and Tfm mice might further elucidate this as androgen receptor signalling is disabled in each case. Reports have shown that in both instances, adults lack any WD-derivatives (Drews and Dieterich, 1978; Quigley *et al*, 1995), however, no definitive evidence is published regarding the status of the fetal WD. This is further discussed in chapter 6.

In controls, WDs showed a significant age-dependent increase in coiling, a morphological sign of differentiation, as determined by quantification of the WD luminal length: this is believed to be an androgen dependent process. Unsurprisingly, flutamide partially impaired this differentiation, as seen by the reduced WD luminal length in fetuses from dams exposed to flutamide (50 or 100 mgkg⁻¹). This confirms the vital role for androgens in differentiation of the stabilised WD. The reduction in coiling was more pronounced in WDs from pups from high dose flutamide litters than from low dose litters, suggesting that WD differentiation is dependent not only on the presence of androgens, but also on the level of androgen action.

At e19.5, WDs from all flutamide-exposed animals were morphologically intact suggesting that AR-mediated signalling in the WD was incompletely blocked in the fetus, even at 100 mgkg⁻¹ flutamide, thus allowing the WD to be rescued. This dose of flutamide is approximately 4-fold higher than that required to prevent prostate development and to completely feminise the external genitalia (Imperato-McGinley *et al*, 1992). This difference is likely due to the much higher local concentrations of testosterone reported in the WD than in tissues derived from the urogenital sinus (Veyssiere *et al*, 1982). It is worth noting that exposure to flutamide does not reduce fetal testicular testosterone levels at e21.5 but actually causes a slight increase in testosterone production (unpublished data). In adults from dams that had been similarly exposed to 100 mgkg⁻¹ flutamide, the majority of the WD-derived tissues were largely absent in 83% of adult males examined. This is in agreement with previous studies showing that exposure to anti-androgenic compounds *in utero* results in a high frequency of epididymal malformations in adult rats (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1999; McIntyre *et al*, 2001; Turner *et al*, 2003; Foster and Harris, 2005). The contrast between the prevalence of flutamide induced WD abnormalities at e19.5 (0%) and in adulthood (83%) demonstrates that the major effect of flutamide is on epididymal differentiation rather than its stabilisation, contrary to suggestions by earlier researchers (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1999). This increased prevalence of epididymal abnormalities with age may be a result of failure to establish normal patterning of the WD fetally. Interfering with androgen action within a critical window of development can therefore impair WD patterning and hence differentiation into its adult derivatives, resulting in irrecoverable malformation of the reproductive tract and likely impairment of fertility. Further studies were undertaken to investigate this window for androgen action during WD development and will be discussed in chapter 5.

By e21.5, WDs from flutamide-exposed animals showed abnormalities in both gross morphology and histology, including shorter, flatter epithelia, occasional missing corpus

segments of the future epididymis, reduced coiling and incomplete lumens. The widened lumens seen in some animals at e21.5 may be due to the collapse of the lumen distally, resulting in blockade of fluid flow down the duct. These abnormalities were not seen until e21.5, thus the epithelium initially forms normally in flutamide-exposed animals but degenerates during differentiation, possibly due to interrupted androgen-driven signalling between the stroma and epithelium. This epithelial flattening was also reported by previous studies in which animals were exposed to Di-*n*-butyl phthalate (DBP; 500 mgkg⁻¹) during fetal life but flattening of the epithelium was not observed until pnd16 (Barlow and Foster, 2003). Furthermore, castration of fetal rats on day 19 resulted in epithelial cells changing shape from columnar to more cuboidal cells (Inomata *et al*, 1989). In multiple organ systems, it is believed that steroid hormones control the fate of epithelial cells via interactions with the underlying stroma and hormone withdrawal can result in epithelial-mesenchymal transformation (Donjacour and Cunha, 1991; Cunha *et al*, 1992; Cunha and Young, 1992; Cunha *et al*, 1996; Aupperle *et al*, 2004). Since the epithelium in incomplete regions often appeared flattened with some cells even looking more fibroblast-like, it may be that a lack of androgen signalling resulted in de-differentiation of some epithelial cells into mesenchymal-like cells thus contributing to the impaired development of the WD. These missing segments were only noted in the corpus segment, the region of the WD that coils last and the segment of the adult epididymis that is least coiled. The 'androgen signal' may be weaker in the corpus than in the caput or cauda and thus flutamide treatment could have more impact on its development. The caput is closest to the testis and is therefore likely to be exposed to high levels of locally delivered androgens, but it is not obvious why the cauda should be less affected by flutamide treatment than the corpus. It is possible that the cauda may obtain testosterone from the blood as well as directly from the testis. However, there is unlikely to be a role for the more potent androgen, DHT, as Wilson (Wilson and Lasnitzki, 1971) and Siiteri (Siiteri and Wilson, 1974) both reported that DHT was not detectable in the WD until after epididymal differentiation was complete.

The most obvious explanations for the abnormal WD development are an increase in cell apoptosis and/or a decrease in cell mitosis. Since apoptosis was minimal in WDs from flutamide-exposed animals, it was considered likely that altered cell proliferation was responsible. Proliferation was apparent in both the epithelial and stromal cell compartments in WDs from both control and flutamide-exposed (50 or 100 mgkg⁻¹) litters. However, it was clear from these studies, that maternal exposure to flutamide resulted in reduced proliferation in both the epithelial and stromal cell compartments of the WD at all ages examined, although significant effects on stromal cell mitosis were noted earlier than

epithelial effects. This highlights the impact of androgen blockade on cell mitosis in the differentiating WD, showing that androgen regulates both stromal and epithelial cell proliferation but the consequences of reduced androgen receptor signalling can be noted earlier in the stromal compartment.

3.5 Conclusion

There is substantial literature on the effects of reduced androgen action on the male reproductive tract using knockout mice and androgen receptor antagonists, however most have studied the effects in adult males, and few have looked at how this impacts on development in fetal life. These studies have utilised a model that allowed manipulation of androgen action during fetal life, using maternal exposure to 50 or 100 mgkg⁻¹ flutamide. The results of this maternal treatment were comparable to those previously published (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1999; McIntyre *et al*, 2001).

The flutamide regime used in these studies interfered with normal WD development; however, it did not prevent stabilisation of the WD but impaired its subsequent convolution and differentiation into its adult derivatives. This highlights WD development as a 2-phase process with WD stabilisation apparently requiring lower androgen action than WD differentiation. Why WD stabilisation is not affected by administration of high doses of flutamide, approximately 4 fold higher than levels that prevent formation of the prostate and induce feminisation of the external genitalia, is something of a mystery, and merits further study. Flutamide exposure interfered with WD differentiation by reducing cell proliferation rather than inducing cell apoptosis. This was noted in both the epithelial and stromal cell compartments at all ages studied.

Future studies will focus on utilising the present model system to investigate possible mechanisms involved in WD patterning and differentiation.

4 Impact of interrupted androgen action on normal markers of Wolffian duct development and its cell compartments and cytoskeleton

4.1 Introduction

The results in the previous chapter confirmed the essential role for androgens in WD stabilisation and differentiation, since exposure to flutamide (50 or 100 mgk⁻¹) between e14.5-21.5 impaired convolution and differentiation of the WD. Surprisingly, this flutamide regime did not interfere with the earlier stabilisation of the WD, highlighting WD development as a bi-phasic process with stabilisation apparently requiring lower androgen action than does differentiation. The studies presented in this chapter will utilise this model system to investigate the possible mechanisms involved in androgen-dependent WD patterning and differentiation and their perturbation by maternal exposure to flutamide.

Sexual differentiation of the reproductive tract is dependent on hormonal regulation but it is not just the secretion of these key hormones which is vital for normal sexual differentiation, but also the expression of their appropriate receptors in the target tissue. It has been suggested that the pattern of steroid hormone receptor expression reflects the ability of the tissue to respond to that hormone (Bentvelsen *et al*, 1995). There is no evidence for a role for either oestrogens or DHT in WD development (discussed in section 1.6 and 1.5.1.1, respectively) but all evidence points to a vital role for testosterone. Several studies have mapped AR expression in the fetal reproductive tract, highlighting that ARs are initially expressed in the rat WD mesenchyme from at least e16.5 with expression not appearing in the WD epithelium until later, around e18.5, even after the initiation of differentiation. (Bentvelsen *et al*, 1995; Majdic *et al*, 1995) (section 1.5.1.2). This led to the hypothesis that androgens induce epithelial differentiation in the male UGT by acting on the surrounding mesenchyme, but the mechanisms behind such paracrine interactions are still only superficially understood (Cunha, 1976) (discussed in section 1.7).

In both fetal and adult life, the organs of the male and female reproductive tract are composed of both epithelium and surrounding supporting stromal cells. Differentiation of the epithelial cells depends on signals received from the underlying mesenchyme. This patterning and compartmentalisation of reproductive organs varies between different structures in the male reproductive tract but is dependent on androgen action in all structures. It is likely therefore that impaired androgen action may interfere with this patterning, compartmentalisation, and differentiation therefore contributing to the abnormal

development of the WD. For example, Hayward and colleagues showed that as mesenchymal cells in the prostate mature and differentiate into smooth muscle cells, expression of early mesenchymal markers such as vimentin decreased while muscle cell markers such as smooth muscle actin and desmin increased: this is believed to be dependent on androgen action (Hayward *et al*, 1996).

It has been suggested from studies in other reproductive tissues that the extracellular matrix (ECM) and cytoskeleton are likely to be involved in mediating mesenchymal-epithelial signalling and thus promote organogenesis and maintain tissue integrity (section 1.8.1 and 1.9) (Cunha *et al*, 1985). The ECM surrounds cells and plays a role in controlling their behaviour, proliferation and differentiation by regulating the availability of signals, such as growth factors, reaching their cell surface (Streuli and Gilmore, 1999). Little is known about the composition of the ECM and basement membrane in the normal WD or its role in androgen-dependent WD development though studies have suggested a role for them in the regressing female MD (section 1.3.1.1). MD regression is characterised by a disruption of the basement membrane, so allowing direct contact between the epithelium and mesenchyme, thus leading to epithelial degeneration (Dyche, 1979; Ikawa *et al*, 1984; Inomata *et al*, 1989; Austin, 1995). This is hypothesised to be mediated by the ECM (Dyche, 1979; Inomata *et al*, 1989; Austin, 1995). It is feasible therefore that the ECM and basement membrane may play a vital role in preventing degradation of the male WD by mediating androgen signalling between the stroma and epithelium.

Previous studies have shown that reduced androgen action in fetal life results in a loss of epididymal and vas deferens tissue by adulthood, but none of these studies have addressed the mechanisms behind this degradation (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1998; McIntyre *et al*, 2000). Studies reported in chapter 3 have shown that this is not due to impaired WD stabilisation but is due to a bi-phasic degradation of this tissue late in fetal life or post pubertally (section 3.3.4.1.3). It is not understood why this degradation occurs or what mechanisms are involved in mediating this. Studies involving exposure of neonatal rats to DES may provide some insight into potential mechanisms as DES exposure also results in a loss of WD-derived tissue in adults and this was shown to be at least partially mediated by an infiltration of immune cells into the lumen (Atanassova *et al*, 2005). Furthermore, matrix metalloproteinases (MMPs) are known to play a role in degradation of the ECM and have been shown to be involved in MD regression, therefore these enzymes may also contribute to the degradation of the WD-derivatives (Roberts *et al*, 2002).

4.1.1 Aims

To investigate steroid receptor expression in the developing WD and the impact of flutamide treatment on this expression in the WD and its adult derivatives. To establish if altered expression of AR plays a role in flutamide-induced inhibition of WD development.

To investigate differentiation of the specific cellular compartments of the WD and its adult derivatives, both in normal and flutamide-exposed animals. To establish if the effects of flutamide treatment are first detectable in the stromal compartment, consistent with the view that epithelial development is mediated by androgen effects on the stroma.

To examine the extracellular matrix (basement membrane, intermediate filaments and cytoskeleton) in the developing WD and its integrity after exposure to flutamide.

To investigate possible proteins involved in the loss of segments of the WD and its adult derivatives after flutamide exposure.

To examine the role for patterning genes in the normal development of the WD and any alterations to this patterning caused by exposure to flutamide.

4.2 Methods

4.2.1 *In utero* treatments

As detailed in chapter 2, section 2.2, pregnant dams were randomly allocated to treatment groups and dosed once daily by gavage with 1 ml kg⁻¹ maternal bodyweight of the following treatments. Dams were dosed between e15.5-e21.5, or until the day prior to cull if this was before e21.5.

Flutamide: 50mg kg⁻¹ in 1ml corn oil/2.5% DMSO (n=24 litters)
 100mg kg⁻¹ in 1ml corn oil/2.5% DMSO (n=23 litters)
Control: 1ml corn oil/2.5% DMSO (n=68 litters)

Throughout this chapter, offspring from dams treated with flutamide during gestation will be referred to as “flutamide-exposed” or treated “*in utero*”. This does not mean that fetuses or WDs were directly exposed to flutamide but were exposed indirectly via the pregnant dam.

4.2.2 Tissue collection

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation and fetuses were recovered at e17.5 – e21.5. Postnatal animals, older than pnd10, were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Fetuses were killed by decapitation then reproductive tracts were recovered by microdissection and photographed using a Leica ICA camera (section 2.3.2.1). Reproductive tracts and gonads were collected from postnatal animals. Tissue was either fixed in Bouin’s or PFA or frozen (section 2.4).

4.2.3 Immunohistochemistry

Immunohistochemistry was performed on isolated reproductive tracts fixed in Bouin’s, using standard avidin peroxidase protocols in order to highlight the location of proteins of interest, as detailed in section 2.6.1.

4.2.3.1 Non-fluorescent immunohistochemistry

Briefly, sections were cut from paraffin blocks and mounted on glass slides. Sections were then dewaxed and rehydrated then pressure cooked in citrate buffer if required (Table 4.1). Non-specific binding was blocked before adding the appropriate primary antibody overnight, as detailed in Table 4.1. A biotin labelled secondary antibody was used that had been raised against a species-specific sequence on the primary antibody. This biotinylated secondary

antibody signal was amplified by incubation with avidin-biotin conjugated with horseradish peroxidase and the signal was localised using DAB. Sections were counterstained with haematoxylin and mounted with glass coverslips. Further detailed protocols can be found in section 2.6.1.

Table 4.1 Summary of primary antibodies used for immunohistochemistry.

Target Antigen	Source	Dilution	Retrieval	Host Species
Androgen Receptor	Santa Cruz	1:50	Citrate	Rabbit
ER α	Novocastra	1:20	Citrate	Mouse
ER β	Gift from P.Saunders			
5 α reductase I	Gift from B. Robaire	1:35	None	Rabbit
5 α reductase II	Gift from B. Robaire	1:35	None	Rabbit
p63	Santa Cruz	1:500	Citrate	Mouse
Smooth Muscle Actin	Sigma	1:10000	None	Mouse
Pan-cytokeratin	Sigma	1:200	Citrate	Mouse
Desmin	DAKO	1:400	Citrate	Mouse
Vimentin	DAKO	1:1000	Citrate	Mouse
Laminin	Abcam	1:100	Citrate	Rabbit
E-cadherin	BD Biosciences	1:200	Citrate	Mouse
Fibronectin	Abcam	1:100	Citrate	Mouse
MMP-2	In house	1:200	None	Mouse
N-cadherin	Zymed	1:500	Citrate	Mouse
CD45	BD Bioscience	1:50	Citrate	Mouse
CD68	DAKO	1:500	Citrate	Mouse
Neutrophil Elastase	DAKO	1:500	None	Sheep

4.2.3.2 Fluorescent immunohistochemistry

Fluorescent immunohistochemistry was used in some cases to allow co-localisation of more than one antigen simultaneously (see section 2.6.1.3).

4.2.3.2.1 Triple immunohistochemistry protocol

In order to delineate stromal and epithelial compartments, fluorescent immunohistochemistry was used to co-localise three proteins in WDs recovered from control and flutamide-exposed fetuses at e17.5 – e21.5 and in postnatal epididymides (Fig. 4.1). Sections were deparaffinised, rehydrated and antigen retrieval performed as detailed in section 2.6.1. All washes between antibody or reagent incubations comprised of two 5 min washes at room temperature in PBS (Sigma). At each stage, control sections were incubated with blocking serum without antibody in order to confirm antibody specificity. Non-specific binding sites were blocked by incubating sections in normal goat serum (NGS; Autogen Bioclear UK Ltd, Wiltshire, UK) diluted 1:4 in PBS containing 5% bovine serum albumin (BSA; Sigma). Sections were incubated overnight at 4°C with anti-pan-cytokeratin antibody (Sigma) diluted 1:200 in NGS/PBS/BSA. Pan-cytokeratin localisation was detected by incubating sections for 1 h with goat anti-mouse secondary antibody directly conjugated with alexa fluor 488 (Molecular Probes) diluted 1:200 in PBS to produce green fluorescence. Sections were incubated for 30 min with mouse IgG (Sigma) diluted 1:2000 in NGS/PBS/BSA to block any remaining mouse IgG sites and prevent the second mouse antibody sticking non-specifically. Non-specific binding sites were blocked again by incubating sections with NGS/PBS/BSA for 30 min before incubating overnight at 4°C with anti-AR (Santa Cruz) diluted 1:50 in NGS/PBS/BSA. AR immunostaining was detected by incubating sections with biotinylated goat anti-rabbit IgG secondary antibody (DAKO) diluted 1:500 in NGS/PBS/BSA for 30 min followed by incubation for 1 h with streptavidin-conjugated alexa 546 (Molecular Probes), producing red fluorescence. Non-specific binding sites were blocked again by incubating sections with NGS/PBS/BSA for 30 min before incubating overnight at 4°C with anti-smooth muscle actin antibody (SMA; Sigma) diluted 1:500 in NGS/PBS/BSA. Sections were incubated for 1 h with goat anti-mouse labelled with Cy5 (Amersham Biosciences, Little Chalfont, UK) diluted 1:60 in PBS, producing blue fluorescent SMA immunostaining. Sections were counterstained for 10 min with a nuclear-specific blue fluorescent label (Dapi; Sigma) diluted 1:1000 in PBS. Slides were then washed in PBS and mounted in Mowiol mounting medium (Calbiochem; Lutterworth, UK). Fluorescent images were captured using a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

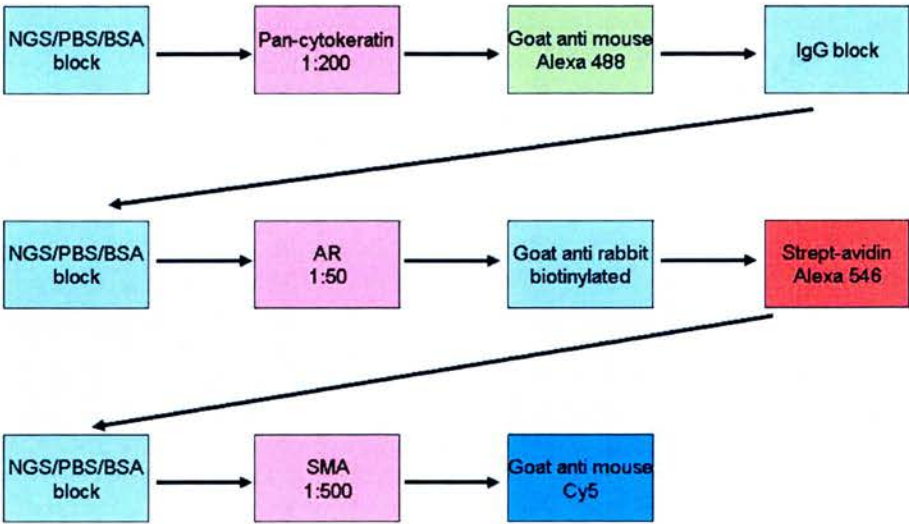


Figure 4.1 Diagrammatic representation of triple fluorescent immunohistochemistry protocol

4.2.3.2.2 Co-localisation of vimentin and cytokeratin

Sections were deparaffinised, rehydrated and antigen retrieval performed as detailed in section 2.6.1. Non-specific binding sites were blocked by incubating sections in NGS/PBS/BSA for 1 h then incubated overnight at 4°C with anti-pan-cytokeratin antibody (Sigma) diluted 1:200 in NGS/PBS/BSA. This signal was detected by incubating sections for 1 h with goat anti-mouse secondary antibody directly conjugated with alexa fluor 546 (Molecular Probes) diluted 1:200 in PBS to produce red fluorescence. Sections were washed well in PBS before blocking in NGS/PBS/BSA for 1 h. Since the anti-vimentin antibody (Dako) and the pan-cytokeratin antibody were both raised in mice, Mike Millar from our histology lab directly labelled the anti-vimentin antibody with Alexa fluor 488 using an Alexa fluor 488 labelling monoclonal antibody labelling kit (Molecular Probes), for which I am extremely grateful. This antibody was diluted 1:15 in NGS/PBS/BSA and incubated on sections for 2 h at room temperature. As no secondary antibody detection was required, slides were washed well in PBS and counterstained with Dapi and mounted as detailed above (section 4.2.3.2.1).

4.2.4 Whole mount immunohistochemistry

Whole mount immunohistochemistry allows localisation of target antigens in a 3-dimensional manner within a whole tissue. This method used similar principles as detailed in section 2.6.1.

4.2.4.1 Re-hydration

After fixation in 4% PFA, fetal reproductive tracts were stored in methanol at -20°C as detailed above (section 2.4.2.1). Prior to immunostaining, the tissue was re-hydrated through a series of methanols in PBS at room temperature. Tissue was incubated in 75% methanol/PBS for 20 min, 50% methanol/PBS for 20 min then 25% methanol/PBS for 20 min. Re-hydrated tissue was washed in PBS for 5 min then in distilled water for 48 h, with several changes. The tissue was then washed in PBS with 0.5% Triton-X (PBSTx; Sigma) for 5 min.

4.2.4.2 Permeabilisation

Fixatives induce cross-linking in order to preserve tissue integrity, but this can often mask proteins (antigens) and thus prevent their immunodetection. In order to unmask these antigens, tissues were permeabilised/digested using 0.05% trypsin (Sigma) in PBS with 0.1% Calcium Chloride. Tissues were incubated for 30 min in the trypsin solution which was pre-heated to 37°C in a waterbath.

4.2.4.3 Non-specific blocking

Tissues were washed in PBS for 5 min prior to incubation for 6 h at room temperature in 2% normal goat serum diluted in PBSTx (PBSTx/NGS). Any non-specific binding of the secondary antibody was prevented by incubating sections in a dilute solution of serum from the species in which the secondary antibody was raised. Triton-X is a detergent therefore aided the unmasking of the antigens and increased the sensitivity of the immuno-detection.

4.2.4.4 Primary antibodies

Blocking buffer was replaced with primary antibody (Table 4.2), diluted in blocking buffer to an optimised concentration. Tissues were incubated on a rotator in a 0.5 ml Eppendorf™ overnight at 4°C with SMA diluted 1:7500 in PBSTx/NGS. Control tissues were incubated with blocking serum only to confirm staining specificity.

Table 4.2 Summary of antibodies used for whole mount immunohistochemistry

Target Antigen	Source	Dilution	Host Species
Smooth Muscle Actin	Sigma	1:7500	Mouse
Pan-cytokeratin	Sigma	1:400	Mouse

4.2.4.5 Secondary antibodies

Tissues were washed well in PBSTx for 2 h at room temperature on a rotator to remove any residual primary antibody thus preventing non-specific background staining. The secondary

antibody was raised against a species-specific sequence on the primary antibody. SMA/cytokeratin was immunodetected by incubating tissues for 3 h with goat anti-mouse secondary antibody directly conjugated with alexa fluor 488 (Molecular Probes) diluted 1:200 in PBS to produce green fluorescence. Tissues were washed well in PBSTx for 1 h at room temperature on a rotator to remove any residual primary antibody

4.2.4.6 Microscopy

WDs were transferred into a 12 well plate (Corning Incorporated, Loughborough, UK) and visualised using a Leica MZFL III microscope (Leica Microsystems). Cellular localisation of SMA was determined and WDs were photographed using a Coolsnap camera (Photometrics, Roper Scientific, Buckinghamshire, UK). WDs were not archived since fluorescence fades within approximately 2 weeks.

4.2.5 Western blots

Western blotting was carried out to separate a mixture of proteins according to their molecular size, as detailed in section 2.6.2. Briefly, protein was extracted from frozen WDs (e17.5–e21.5) using RIPA lysis buffer (section 2.6.2.1) and 15ug of each protein was loaded into an SDS-page gel and subject to electrophoresis at 100 V for 1–2h. Proteins were electro-transferred from the gel and immobilised onto a nitrocellulose membrane. Proteins were subsequently detected on the membranes using antibodies specific to proteins on the membrane (Table 4.3) as described in 2.6.2.5. An anti- β -tubulin antibody (Sigma) was used as a standardization loading control, diluted 1:300 in TBST.

Table 4.3 Summary of primary antibodies used for Western blotting

Target Antigen	Source	Dilution	Host Species
Androgen Receptor	Santa Cruz	1:300	Rabbit
Smooth Muscle Actin	Sigma	1:10000	Mouse
Vimentin	Dako	1:4000	Mouse
β -tubulin	Sigma	1:300	Mouse
E-cadherin	BD Biosceinces	1:2000	Mouse

4.2.6 RNA extraction

RNA was extracted from frozen WDs at e17.5–e21.5 using RNeasy Mini extraction kits (Qiagen, Crawley, UK) and quantified using RNA 6000 Nano chips on an Agilent 2100 Bioanalyser (Agilent Technologies, Wokingham, UK), as detailed in section 2.7.1–2.7.2.

4.2.7 Taqman QRT-PCR

Random hexamer primed cDNA was prepared using the Applied Biosystems TaqMan ® reverse transcription kit (Applied Biosystems, Foster City, CA) (see 2.7.3.2) and quantitative PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Expression of specific gene mRNA was determined using the Assay-On-Demand Gene Expression ™ system (Applied Biosystems), as detailed in Table 4.4. Detailed protocols can be found in section 2.7.5.

Table 4.4 Summary of Taqman ® Assay-On-Demand Gene Expression ™ primes used.

Gene of interest	Assay number
AR	Rn00560747
SMA	Rn00563662
MMP-2	Rn01538167

4.2.8 PCR

Oligo-dT primed cDNA was amplified using Bioscript Reverse Transcriptase (Bioline, Germany) according to manufacturer’s instructions (section 2.7.3.1). Oligonucleotide sequences for Hox A10 primers were designed and generously provided by Griet Vanpoucke: primers were synthesised by MWG-Biotech (Germany). Table 4.5 details the specific sequences, annealing temperature and product sizes for each primer pair used; primers were diluted to 5µM before use.

Table 4.5 Summary of primer pairs used for PCR

Gene	sequence	Product size	Annealing temperature
Hox A10 forward	AGCGAGTCCTAGACTCCAC	500bp	65°C
Hox A10 reverse	AGGCAGAAGGATGGGTACAG	500bp	65°C

4.2.8.1 PCR reaction

In all PCR reactions, Bio Taq (Bioline) was used, according to manufacturer’s instructions as detailed below.

Mastermix:

	Actual Volume	Final
concentration		
10x NH ₄ Buffer	1 µl	1x
50mM MgCl ₂	0.3 µl	1.5mM
2mM dNTPs	1 µl	200µM
Forward Primer 5µM	1 µl	0.5µM
Reverse Primer 5µM	1 µl	0.5µM
Taq Polymerase (5units/µl)	0.25 µl	1.25 units
H ₂ O	3.45 µl	
cDNA	<u>2 µl</u>	
Total	10 µl	

Reagents were added together in a sterile 0.2ml thin walled PCR tube (Continental Lab Products) and thermo-cycled through the following programme.

Denature - 95°C for 5 min

Amplification (30 cycles):

Denature - 94°C for 30 s

Anneal – 50-58°C (depending on primers) for 30 s

Extension - 72°C for 1 min

Extension - 72°C for 10 min

4.2.8.2 Electrophoresis

4.2.8.2.1 Loading Dyes and Ladders

Once the target gene was amplified using PCR, they were analysed using agarose gels. A loading dye was added to each sample in order to retain the DNA in wells and prevent it floating away. This dye also allows visualisation of how far each sample has run on the gel. Different dyes will run at different speeds due to differences in the size of the molecules, however, Orange G was used in all experiments. Orange G (50% 1x TAE, 20% glycerol, 0.25% Orange G) runs to 50 base pairs (bp) therefore products will still be on the gel if they are bigger than 50bp. Each sample had 5µl Orange G added to it before being loaded into an agarose gel (see details below). A DNA ladder was also run on each gel to check that PCR products were the expected size and confirm amplification of the correct target sequence. Hyperladder IV (Bioline), a DNA ladder with loading buffer already added, was used. This allows accurate sizing and quantification of DNA between 100bp and 1000bp.

4.2.8.2.2 Agarose gel

PCR products were electrophoresed on 2% agarose gels (150 ml 1x TAE buffer + 3 g Agarose (Bioline)). This solution was heated in the microwave to ensure agarose was dissolved. This was allowed to cool slightly then 75 μ l ethidium bromide (1mgml⁻¹ at 1/2000) was added to the agarose solution: ethidium bromide intercalates between the DNA bases and fluoresces under UV light to allow visualization of the DNA bands in the gel. The agarose gel solution was poured into a case with a comb to form the wells and was then left to cool and set.

4.2.8.2.3 Electrophoresis and visualisation

The agarose gel was transferred into an electrophoresis tank (Biorad, Hertfordshire, UK) filled with 1x TAE buffer. The DNA ladder and PCR samples were loaded into wells and the electrodes were connected up to a Biorad power pack 300 (Biorad) and subjected to 75V for approximately 1 h. Gels were visualized using a transilluminator.

4.3 Results

4.3.1 Expression of steroid receptors in the Wolffian duct and its adult derivatives

4.3.1.1 Androgen receptor expression

4.3.1.1.1 Androgen receptor expression in the fetal Wolffian duct

The androgen receptor (AR) was immunolocalised to cell nuclei throughout the WD at all ages examined (e16.5 – e21.5). AR expression was first detected in a few sporadic cells in the WD stroma at e15.5 and in most stromal cells by e16.5: epithelial expression did not switch on until around e17.5 (Fig. 4.2). In all subsequent fetal ages, inner stromal cells directly surrounding the epithelium showed the most intense immunostaining while the epithelial cells showed weaker staining at all ages (Fig. 4.2). WDs from flutamide-exposed animals displayed a similar pattern of AR expression to controls at all ages studied with more intense AR expressed in the stroma (Fig. 4.3) and epithelial expression switching on in some cells by e17.5 (Fig. 4.3). This differing expression in the inner stroma compared to the epithelium can be highlighted by co-staining AR with cytokeratin, an epithelial cell marker, and SMA, a marker of inner stroma (Fig. 4.4).

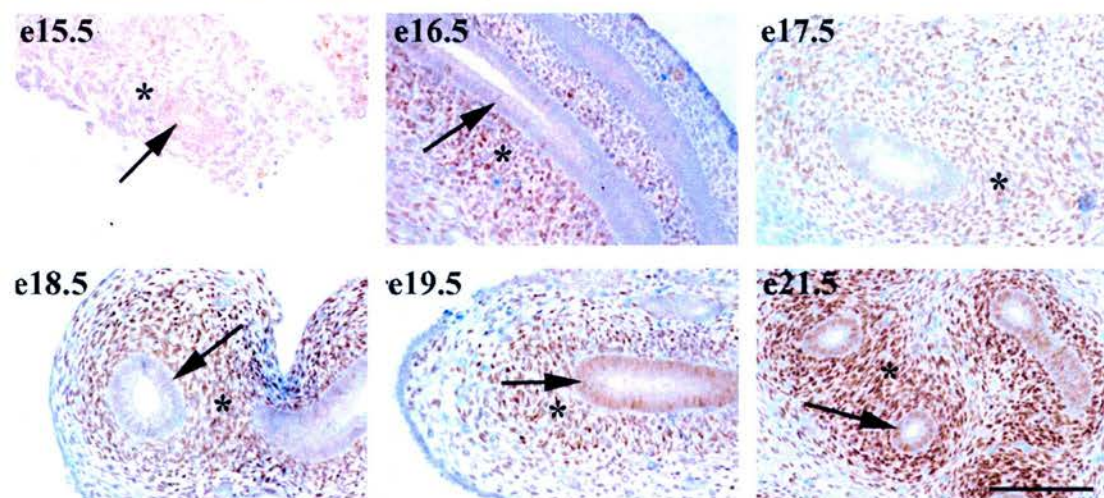


Figure 4.2 *Immunoexpression of androgen receptor (AR) in the developing Wolffian duct (WD) from e15.5-e21.5. AR was not detected in the WD at e15.5 but was expressed in the WD stroma by e16.6 (immunostained brown, *). AR expression in the epithelium (arrow) began to switch on around e17.5 and was strongly expressed in the epithelium by e19.5. AR was intensely expressed in both the stroma (*) and the epithelia (arrow) by e21.5. Scale bar = 100 μ m*

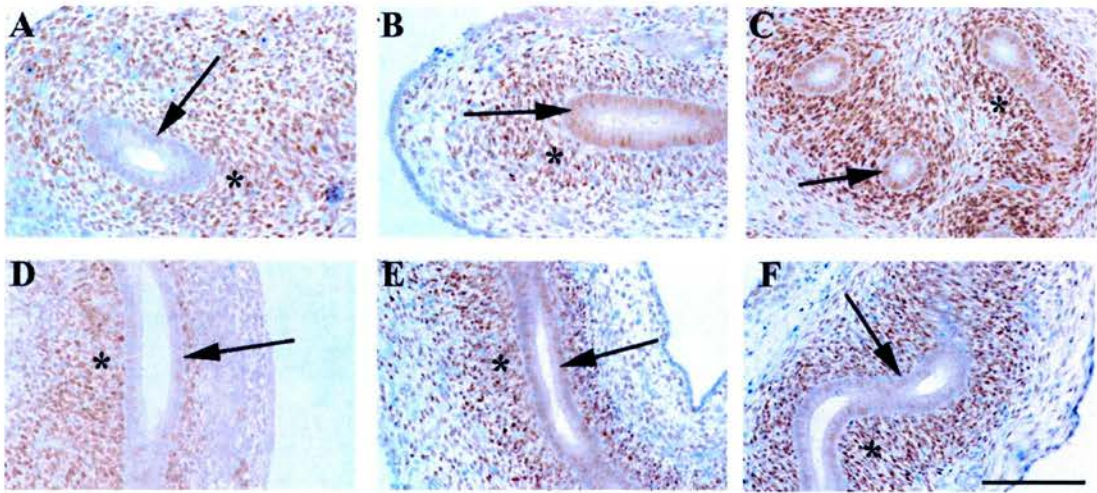


Figure 4.3 Expression of androgen receptor (AR) protein in the Wolffian duct (WD) from control (A-C) and flutamide-exposed (D-F, 100 mgkg^{-1}) rats at e17.5 (A & D), e19.5 (B & E) and e21.5 (C & F). The pattern and timing of AR expression did not vary between control and flutamide-exposed WDs: stromal AR (*) was strongly expressed at e17.5 with epithelial AR expression (arrow) switching on at around e17.5 in flutamide-exposed WDs (D). Scale bar = 100 μm

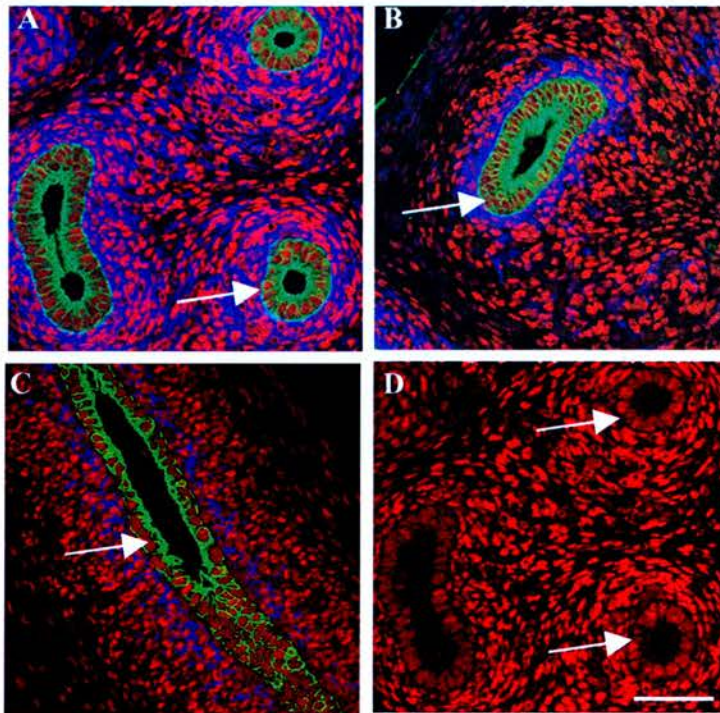


Figure 4.4 Androgen receptor (AR) expression in the different Wolffian duct (WD) compartments at e21.5. Note that the inner stroma is demarcated by smooth muscle immunostaining (SMA; blue) and the epithelium by cytokeratin staining (arrow, green). No obvious difference was seen in AR expression (red) between WDs from control animals (A) and animals exposed to 50 mgkg^{-1} (B) or 100 mgkg^{-1} (C) flutamide. Image D shows AR only staining from Fig 5A, highlighting that AR protein is expressed more intensely in the stroma

than in the epithelium (arrows) of the control WD at e21.5 (D) and flutamide treatment had no obvious effect on this pattern (B & C). Scale bar = 50µm

Further analysis of AR expression using Western blotting and Taqman QRT-PCR confirmed that there was no significant difference in either AR protein (Fig. 4.5) or mRNA (Fig. 4.6) expression in control compared to flutamide-exposed WDs at e18.5-e21.5. Variation was seen in the levels of AR mRNA and protein within each age/treatment however, this was not significant and presumably represents variation between individual WDs.

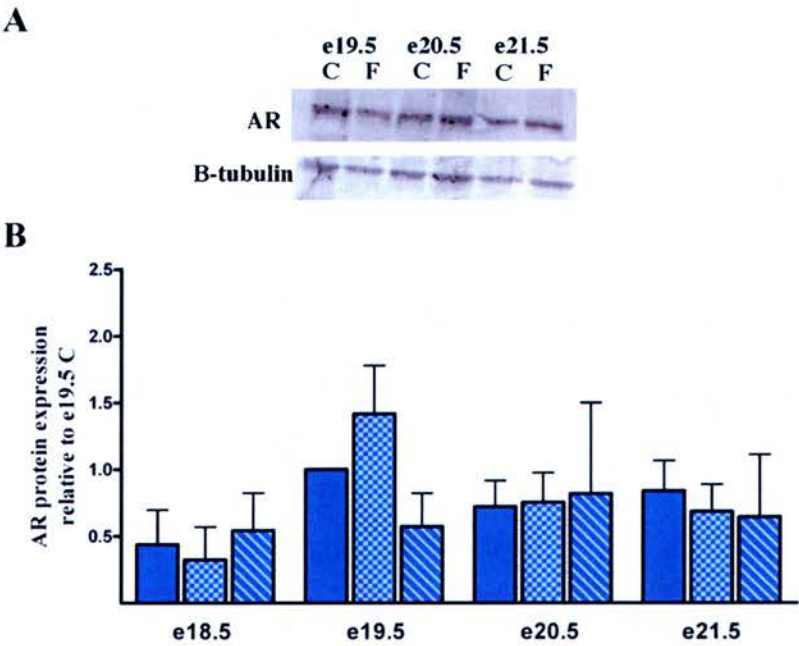


Figure 4.5 Quantification of AR protein levels in the Wolffian duct (WD). Panel A shows a representative Western blot for AR protein expression in WDs at e19.5 – e21.5 (n=3 WDs per age/treatment) with its loading control below. Panel B shows quantitative analysis (mean ± SEM) of AR protein from Western blots confirming no significant change in AR expression in WDs at e18.5-e21.5 from control (solid blue bars) and flutamide-exposed (chequered bars - 50 mgkg⁻¹; striped bars - 100 mgkg⁻¹) rats (n=6 Western blots, each using a pool of 3 WDs per age/treatment, from at least 3 litters). Protein expression was corrected for loading and expressed relative to levels in e19.5 control WD (means ± S.E.M, n=3-5 WDs per age/treatment, each from different litters).

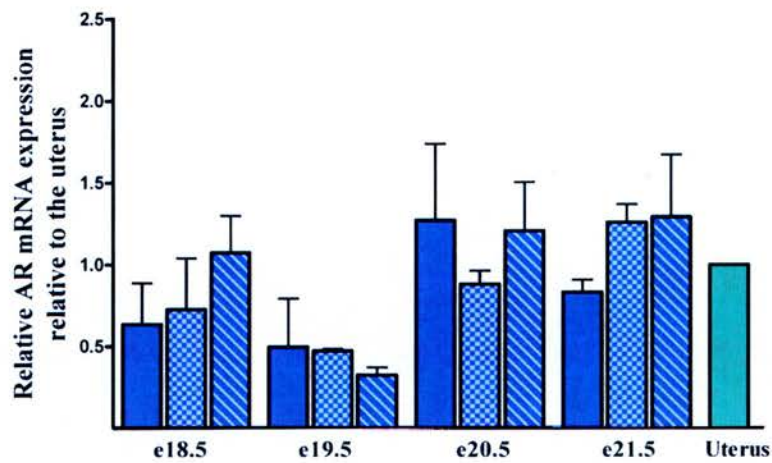


Figure 4.6 Quantitative analysis of AR mRNA in the Wolffian duct. No significant difference was seen in AR mRNA expression in WDs at e18.5-e21.5 from control (solid blue bars) and flutamide-exposed (chequered bars - 50 mgkg⁻¹; striped bars – 100 mgkg⁻¹) rats, as determined by Taqman QRT-PCR (means \pm S.E.M, n=3-5 WDs per age/treatment, each from different litters). mRNA expression was corrected for loading and expressed relative to levels in the uterus.

4.3.1.1.2 Androgen receptor expression in postnatal WD-derived structures

AR continues to be expressed in the epithelium and stroma in postnatal WD-derived structures. The expression pattern alters from the fetal WD with epithelial AR expression becoming more intense than in the stroma (Fig. 4.7, panels A & B). Epididymides and vasa deferentia from males exposed to flutamide *in utero* (100 mgkg⁻¹) continue to express AR in both the stroma and epithelium, but this expression looks slightly reduced (Fig. 4.7, panels C-F). Note that AR is expressed in the epithelium even in areas where the epididymis looks abnormal both at the gross and histological level but it is no longer expressed in every epithelial cell (Fig 4.7, panels E & F).

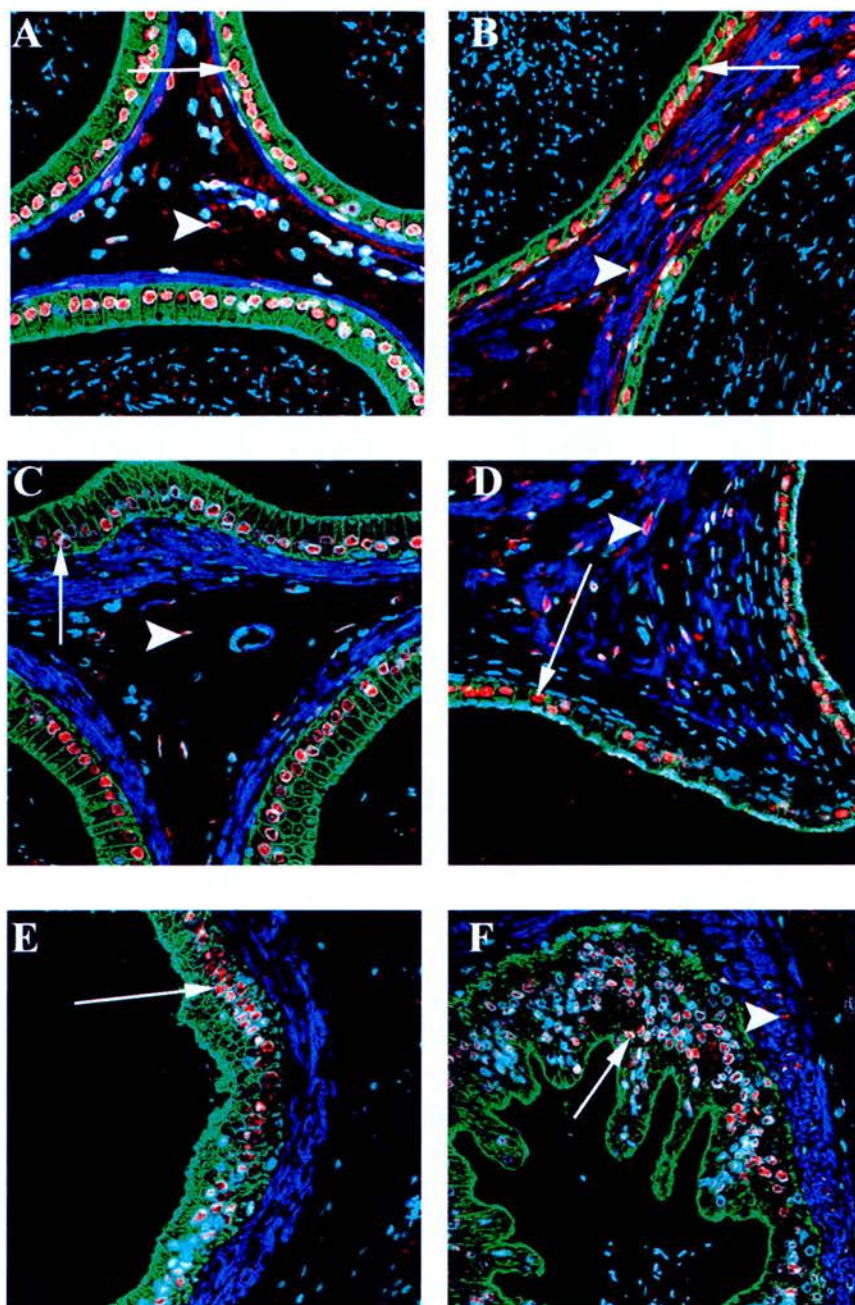


Figure 4.7 Androgen receptor (AR) protein expression in postnatal epididymides from control (A & B) and flutamide-exposed (100 mgkg^{-1} , C-F) animals at pnd70. AR protein (red) is expressed in most epithelial cells (cytoplasm immunostained green for cyokeratin; arrow) and in some stromal cells (arrowhead) in the adult caput epididymis (A) and cauda (B). Note that in some areas of the flutamide-exposed epididymis, the AR expression pattern did not vary from the same region of the control epididymis (C compared to A and D compared to B) while in areas where the epididymis has an abnormal phenotype (E – caput and F – corpus) AR expression varied from controls. Sections were counterstained light blue and the inner stromal smooth muscle layer is stained blue for smooth muscle actin.

4.3.1.2 Oestrogen receptor expression in the fetal Wolffian duct

4.3.1.2.1 Oestrogen receptor α

Oestrogen receptor alpha ($ER\alpha$) was immunolocalised to the efferent ducts, not the fetal WD, in all reproductive tracts studied at e17.5-e21.5 (Fig. 4.8). This expression pattern did not change in flutamide-exposed WDs.

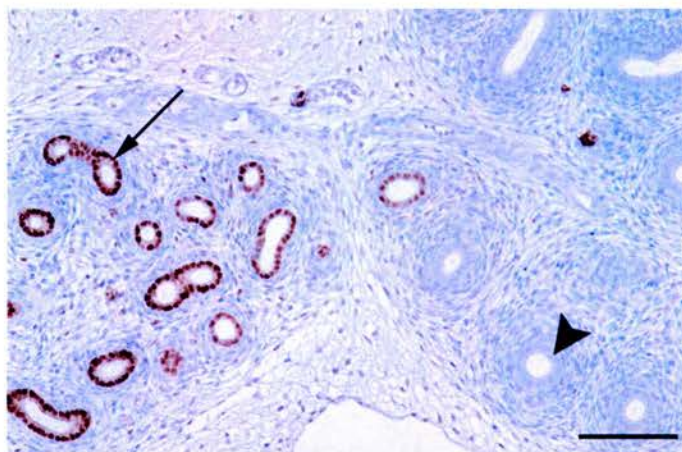


Figure 4.8 Oestrogen receptor α ($ER\alpha$) protein expression in the efferent ducts at e21.5. Note that $ER\alpha$ is only expressed in the efferent duct epithelium (brown, arrow) and was not detected in the Wolffian duct epithelium (arrowhead) or stroma. Scale bar = 100 μ m

4.3.1.2.2 Oestrogen receptor β

Oestrogen receptor β ($ER\beta$) was immunolocalised to both the stroma and epithelium in the WD at all ages studied and showed no obvious variation with flutamide exposure (data not shown).

4.3.1.2.3 5α -reductase type I and type II

5α -reductase type I and type II protein were not detected, by immunohistochemistry, in the fetal WD (data not shown).

4.3.1.3 $RXR\alpha$

Retinoid X receptor α ($RXR\alpha$), a member of the ligand-dependent retinoic acid receptor transcription factor family, was immunolocalised to cell nuclei in both the stromal and epithelial compartments of the WD at all ages studied (e17.5-e21.5). Immunoexpression was less intense in WDs from fetuses exposed to flutamide *in utero* (Fig. 4.9) and was almost absent in e21.5 WDs exposed to 100 mgkg^{-1} flutamide (Fig. 4.9, panel F).

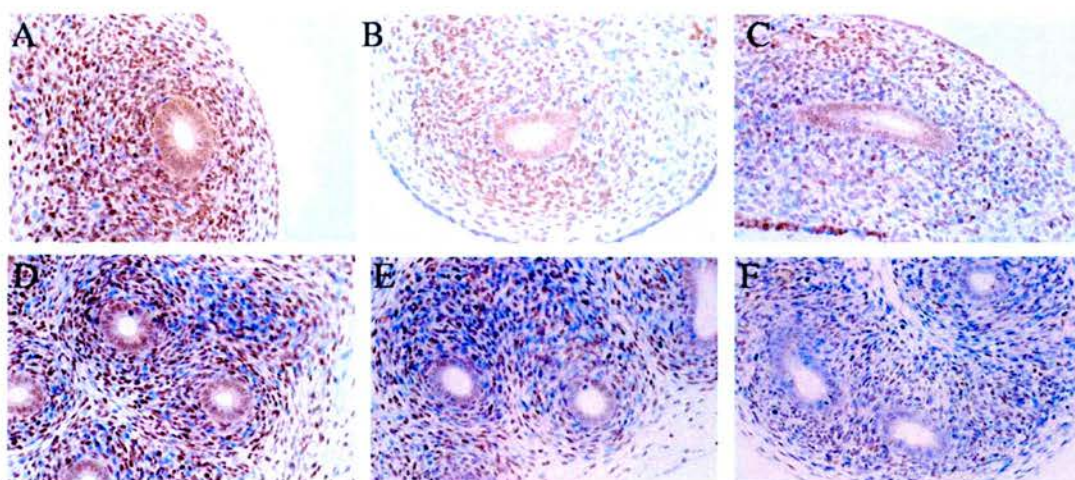


Figure 4.9 *RXR α* expression in the developing rat Wolffian duct at e18.5 (A-C) and e21.5 (D-F). Note that *RXR α* is expressed in both the stroma and the epithelium. At both e18.5 and e21.5 *RXR α* expression is reduced in WDs from flutamide-exposed animals (e18.5 B-50 mgkg⁻¹ and C – 100 mgkg⁻¹; e21.5 E -50 mgkg⁻¹ and F – 100 mgkg⁻¹) compared to their age matched controls (A and C respectively).

4.3.2 Cell differentiation markers

4.3.2.1 Immunoexpression of basal epithelial marker p63

The basal epithelial cell marker p63 was not detected in the WD at any age studied (e21.5-pnd0) but was detected in the epithelium lining the neonatal urogenital sinus at pnd0 (Fig. 4.10). By pnd17, p63 was immunolocalised to the epithelium in WD-derived structures (Fig 4.11) and continued to be expressed throughout postnatal life. p63 was still immunolocalised to the epithelium in postnatal males that had been exposed to flutamide *in utero*, however this expression was reduced compared to age matched controls (Fig 4.11). This reduction became more obvious in adults than in pre-pubertal males with p63 staining only being evident in a few cells in some cross-sections of epididymis while in others there was no obvious p63 expression at all. In some flutamide-exposed males, the epididymal epithelium looked abnormal and was folded into crypt-like structures. In these areas, p63 staining appeared increased and not restricted to the basal cells (Fig. 4.12).

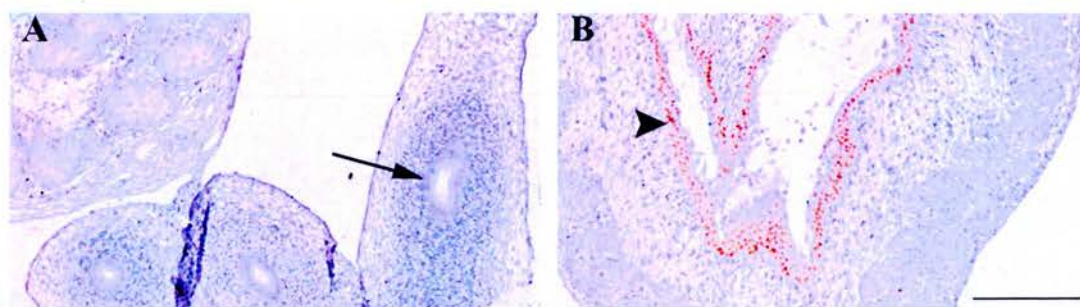


Figure 4.10 *Immunoexpression of p63 in the neonatal reproductive tract (pnd0). Note that p63 was not expressed in the Wolffian duct epithelium (arrow) or stroma (A) but was detected in the urogenital sinus epithelium (brown, arrowhead, B) from the same animal. Scale bar = 100 μ m.*

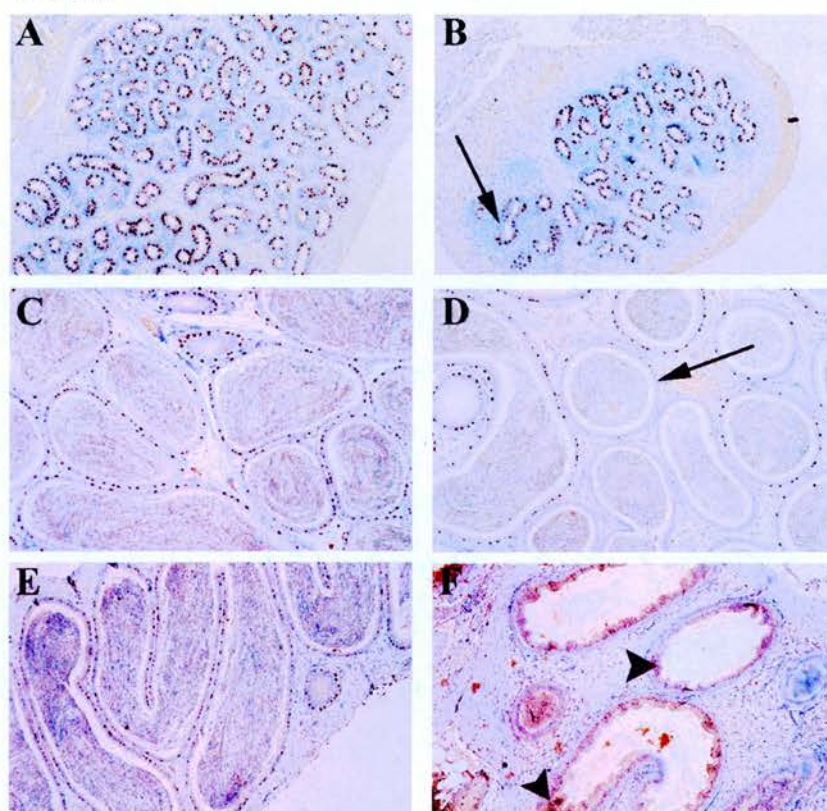


Figure 4.11 *Immunoexpression of p63 protein in the basal epithelial cells in the postnatal epididymis (pnd17 - 70). Note that at pnd17, p63 is strongly expressed in basal epithelial cells in the control epididymis (A) but this expression is slightly reduced in the epididymis from flutamide-exposed animals (arrow, 100 mgkg^{-1} , B). By adulthood (pnd70) p63 continues to be strongly expressed in basal epithelial cells in the control epithelium in the caput (C) and corpus (E) regions but expression is altered in flutamide-exposed epididymides (100 mgkg^{-1} , D and F). Note that in the caput, p63 expression is reduced and often absent in entire cross sections (arrow, D) whereas in the corpus, where the epididymis had an abnormal phenotype, p63 expression is increased (arrowhead, F). Scale bar = 100 μ m*

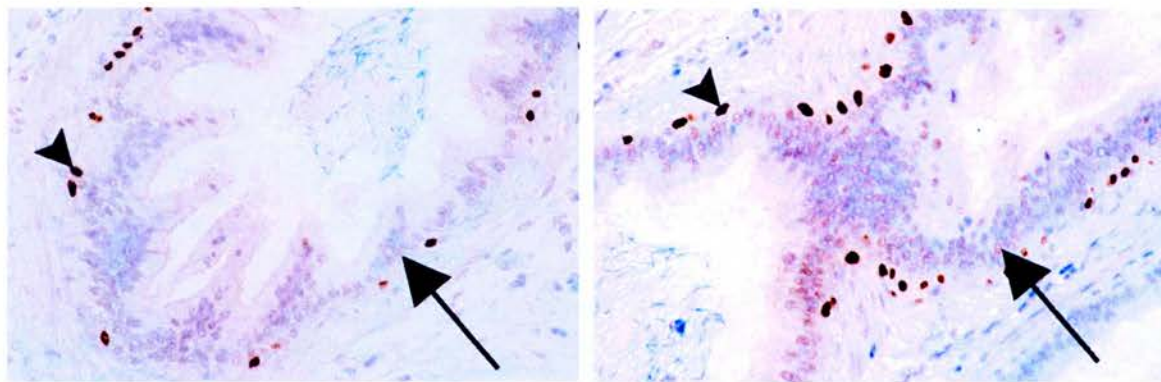


Figure 4.12 Abnormal immunorexpression of p63 in adult epididymis from a male exposed to flutamide (100 mgkg^{-1}) in utero. Note the abnormal morphology of the epithelium with folding into crypt-like structures and multilayers of cells. Note the strong p63 staining in basal cells and the less intense staining in the more apical cells. This was not seen in control adult male epididymis.

4.3.2.2 Smooth muscle actin, a marker of WD stromal differentiation

4.3.2.2.1 Switching on of smooth muscle actin in control fetal WDs

Immunorexpression of smooth muscle actin (SMA), a marker of smooth muscle differentiation, was first evident in the fetal rat WD at e18.5 (Fig. 4.13). SMA was only detected in the stromal cells immediately adjacent to the epithelium, termed here as inner stroma, not in the outer stromal compartment (Fig 4.13). This SMA expression was first evident in the corpus segment of the WD at e18.5 (Fig. 4.13) and progressed towards the caput and corpus by e20.5 (Fig 4.14).

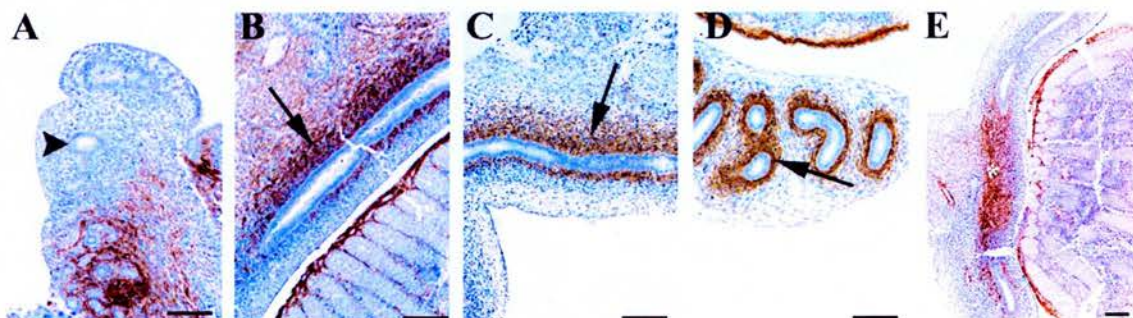


Figure 4.13 Immunorexpression of smooth muscle actin (SMA) in the fetal Wolffian duct (WD) at e17.5-e21.5. Note that SMA is not expressed in the WD at e17.5 (arrowhead, A) but switches on by e18.5 in the stroma immediately adjacent to the epithelium (brown, arrow, B). SMA expression persists in the inner stroma at e19.5 (arrow, C) and e21.5 (arrow, D). Panel E highlights that SMA is first detected only in the corpus region at e18.5 (*), with expression in the caput and cauda regions by e20.5 (not shown). Scale bars = 100 μm

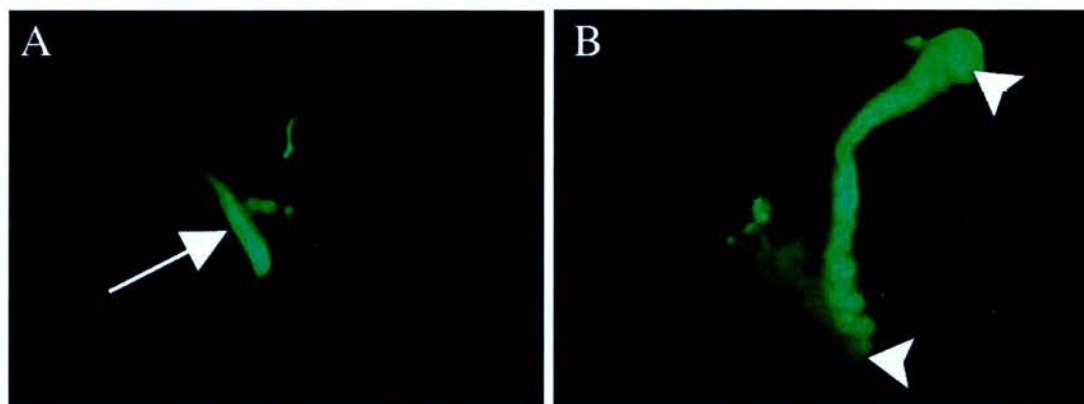


Figure 4.14 Expression of smooth muscle actin (SMA) in the Wolffian duct (WD) at e19.5 (A) and e20.5 (B) using whole mount immunohistochemistry. Note that at e19.5, SMA was detected only in the corpus region (green, arrow, A) but by e20.5, SMA expression had progressed towards the caput and cauda regions (arrowhead, B).

4.3.2.2.2 Evidence for earliest effects of flutamide treatment on the inner stromal cells

As the data presented in chapter 3 (section 3.4.8) indicated an impact of flutamide treatment on both stromal and epithelial tissues of the WD, evidence of stromal-specific effects was sought using smooth muscle actin (SMA), a smooth muscle cell marker specifically expressed in the inner stromal compartment. Immunostaining suggested a reduction in the extent of this SMA positive layer in flutamide-exposed WDs compared to age matched controls, though this varied considerably between WDs (Fig 4.15). This decrease was confirmed quantitatively by Western blot analysis, which revealed a large reduction in SMA protein expression from e18.5 (Fig. 4.15), a time-point at which no epithelial damage or change in luminal length was detectable in flutamide-exposed animals (chapter 3). The reduction in SMA expression continued throughout fetal life.

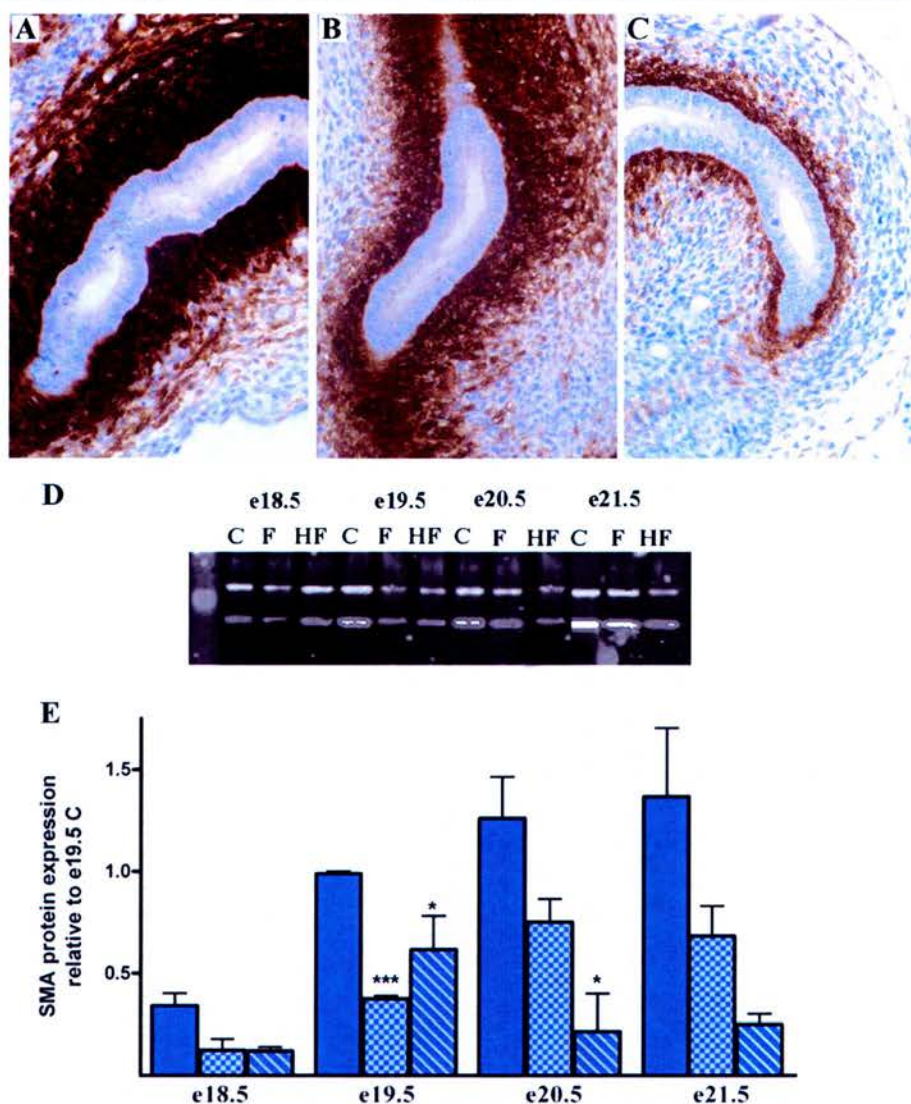


Figure 4.15 Comparative expression of smooth muscle actin (SMA) at e19.5 in Wolffian ducts (WDs) from controls (A) and animals exposed in utero to 50 mgkg⁻¹ (B) or 100 mgkg⁻¹ flutamide (C). Note the reduction in SMA protein expression in WDs after flutamide exposure. Panel D shows a representative Western blot for SMA protein in WDs from control (C) and flutamide exposed animals (F - 50 mgkg⁻¹; HF - 100 mgkg⁻¹) at e18.5-e21.5 ($n =$ a pool of 3-5 WDs per age/treatment, from at least 3 different litters). Panel E shows quantitative data expressed relative to SMA expression in control WDs at e19.5 (corrected for loading using β -tubulin). Note the increase in SMA protein expression between e18.5 and e19.5 in controls (solid blue bars) and the decrease in SMA expression after flutamide-exposure (50 mgkg⁻¹ chequered bars; 100 mgkg⁻¹ striped bars), compared to controls. * $p < 0.05$, *** $p < 0.001$, in comparison with respective control value. Values are means \pm S.E.M for 2-3 Western blots, each using a pool of 3 WDs per age/treatment, from at least 3 litters).

Analysis of SMA mRNA expression showed no consistent or significant difference in mRNA expression with age or flutamide treatment (Fig. 4.16). The fluctuations seen in mRNA expression are likely due to individual variation. It is worth mentioning that SMA expression is not restricted to the WD stroma but is also detected around blood vessels.

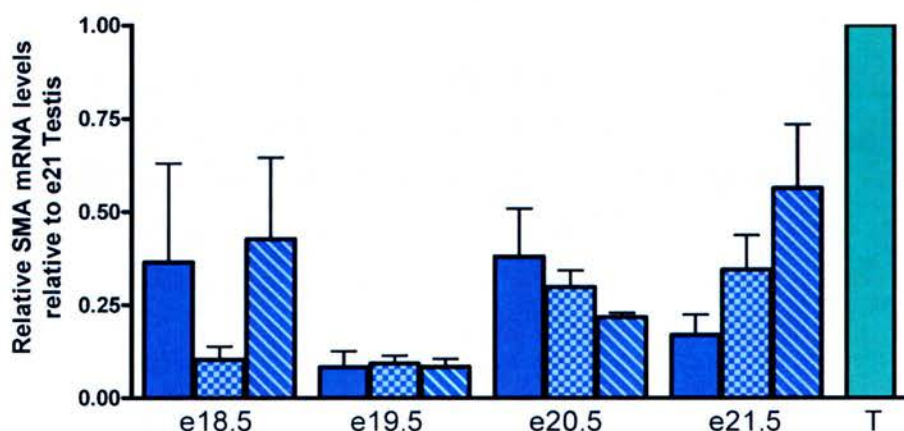


Figure 4.16 Quantitative analysis of smooth muscle actin (SMA) mRNA expression in Wolffian ducts at e18.5-e21.5 in control (solid blue bars) compared to flutamide-exposed animals (50 mgkg⁻¹ chequered bars; 100 mgkg⁻¹ striped bars). Note that no significant difference was seen in SMA mRNA expression with age/treatment, as determined by Taqman QRT-PCR (means \pm S.E.M, n=3-5 WDs per age/treatment, each from different litters). SMA mRNA expression was expressed relative to mRNA levels in control e21.5 testes (green bar, T) and compared to the internal positive control gene expression.

4.3.2.2.3 SMA expression in the postnatal epididymis

SMA can be localised to the stromal cells, immediately adjacent to the epithelium, in the adult epididymis as in the fetal WD (Fig. 4.17). This thin layer of smooth muscle can be seen along the full length of the epididymis, becoming thicker in the vas deferens (Fig. 4.17, panel C and F). Exposure to flutamide *in utero* resulted in a thicker layer of smooth muscle in the adult epididymis, but this varied between individuals and along the length of the epididymis. In the vas deferens, a thin SMA negative layer can be seen between the epithelium and the smooth muscle layer; this negative layer appears to be increased in males exposed to flutamide *in utero* (Fig. 4.17).

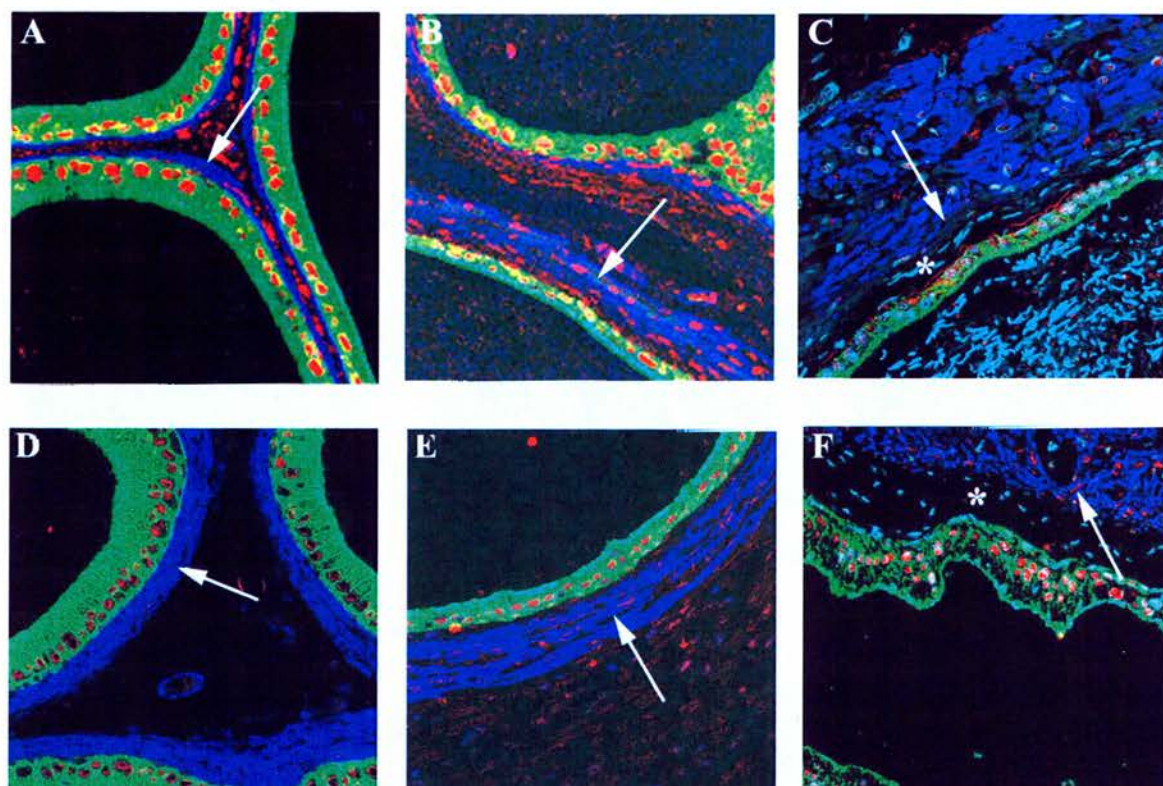


Figure 4.17 Smooth muscle actin immunoexpression (SMA, blue) in postnatal WD-derivatives from control (A-C) and flutamide-exposed animals (100 mgkg^{-1} , D-F) at pnd70. Note that in the caput (A and D) and cauda (B and E) regions of the epididymis, a layer of SMA is seen in the stroma adjacent to the epithelium (arrow) while in the vas deferens (C and F), this SMA positive layer is slightly thicker and a thin SMA negative layer can be seen between the epithelium and the SMA positive layer (*). Note that in the epididymis, the SMA positive layer is slightly wider in the flutamide-exposed animals than in controls. Green = cytokeratin, Red = androgen receptor

4.3.3 Integrity of the cytoskeleton and intermediate filaments in the WD

4.3.3.1 Cytokeratin

Simple columnar epithelia line the lumen of the fetal WD and its adult derivatives. In all ages examined, cytokeratin was expressed in the cell boundary of this epithelium (Fig. 4.18) and its expression did not obviously change with either age or flutamide treatment (50 or 100 mgkg^{-1}). Immunostaining for cytokeratin highlighted abnormalities in the epithelial compartment with cytokeratin still expressed in flattened, damaged epithelium even when it was almost fibroblast-like (section 3.4.5). Cytokeratin localised to the epithelium in the postnatal WD-derivatives, even those with an abnormal phenotype due to exposure to flutamide during gestation (green staining in Fig. 4.16).

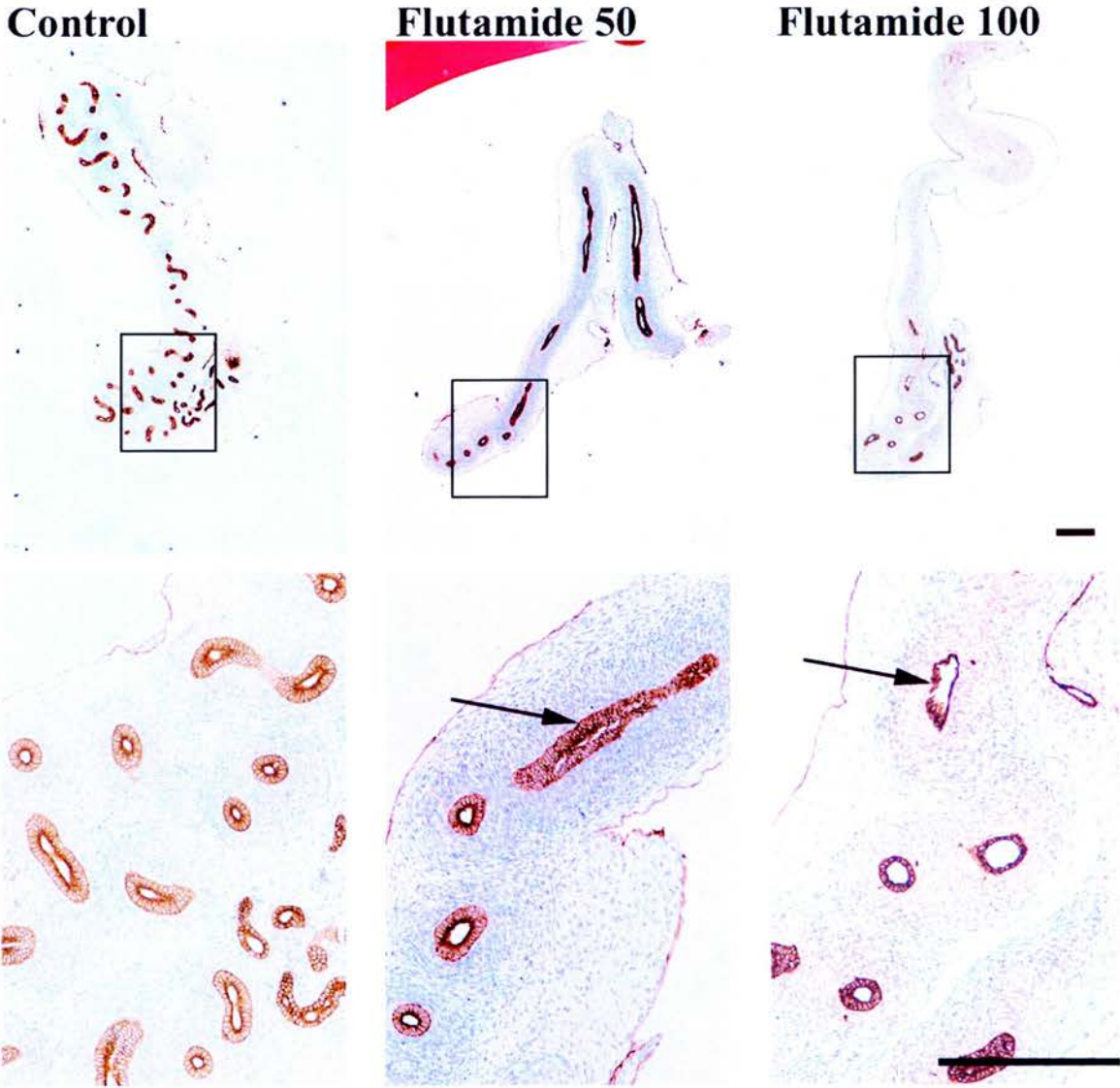


Figure 4.18 Cytokeratin expression (brown) at e21.5 in the Wolffian duct (WD) epithelium from control and flutamide-exposed (50 and 100 mgkg⁻¹) fetuses. Note that cytokeratin is still expressed in epithelia with an abnormal phenotype (arrow). Scale bars = 250 μ m

4.3.3.2 Desmin

Desmin protein was localised to the stromal compartment of the WD from at least e15.5 (Fig 4.19) and continued to be expressed at all ages studied (e15.5-e21.5). Desmin was expressed in both the inner and outer stromal compartments but was never detected in the epithelium. There was no obvious change in desmin expression in flutamide-exposed WD stroma determined by immunohistochemistry (Fig 4.19, C compared to D), and this was confirmed quantitatively by Western blot (Fig. 4.20).

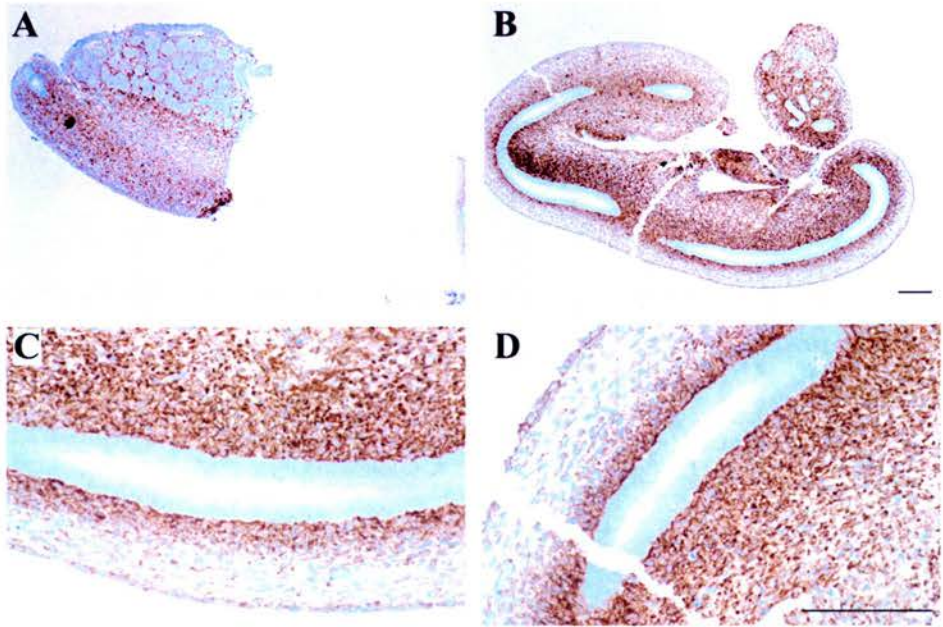


Figure 4.19 Desmin protein expression in the fetal Wolffian duct (e15.5-e18.5). Desmin is detected by e15.5 (brown, A) and continues to be expressed in the WD stroma throughout fetal life (e18.5, B). Note that there is no obvious change in desmin expression in WDs from flutamide-exposed fetuses (100 mgkg⁻¹, D) compared to age matched controls (C). Scale bars = 100 μm

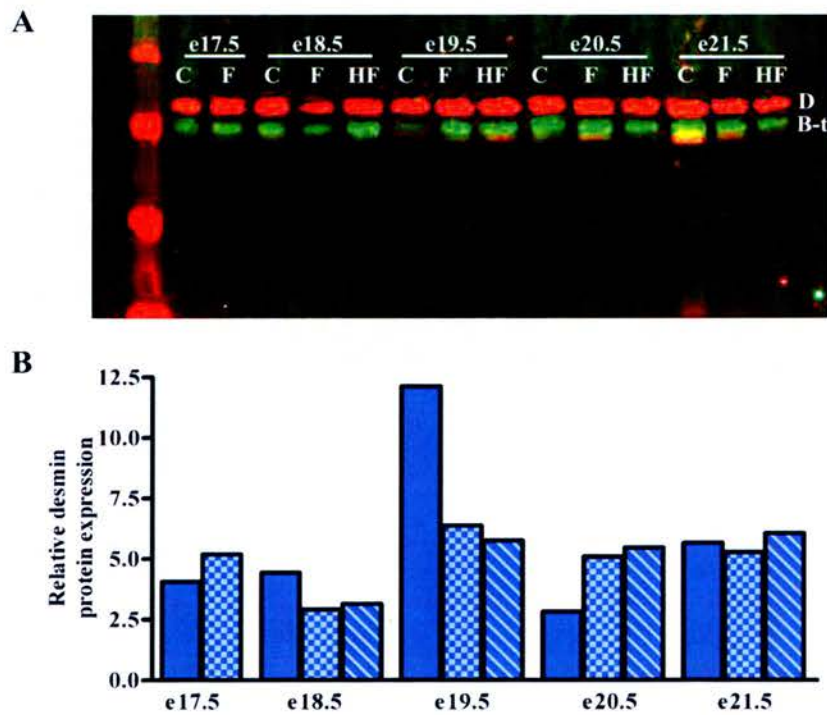


Figure 4.20 Comparative expression of desmin protein at e17.5–21.5 in Wolffian ducts (WDs) from controls and animals exposed in utero to 50 mgkg⁻¹ or 100 mgkg⁻¹ flutamide. Panel A shows a representative Western blot for desmin protein in WDs from control (C) and flutamide exposed animals (F - 50 mgkg⁻¹; HF - 100 mgkg⁻¹) at e17.5-e21.5

($n =$ a pool of 3 WDs per age/treatment, from at least 3 different litters). Panel B shows quantitative data corrected for loading using β -tubulin (B-t). Note that no consistent change was noted in desmin protein expression after flutamide-exposure (50 mgkg^{-1} chequered bars; 100 mgkg^{-1} striped bars), compared to controls. Values are means \pm S.E.M.

4.3.3.3 Vimentin

Vimentin immunolocalised to the WD stroma of both control and flutamide exposed fetuses at all ages examined (e17.5-e21.5; Fig. 4.21). Vimentin is also expressed in the basolateral edges of epithelial cells in the distal corpus and cauda of the future epididymis. This 'spiking' was seen along the full length of the WD at e15.5 and e17.5 but by e19.5, epithelial expression became mainly restricted to the cauda and was rarely seen in the caput epithelium in control WDs (Fig. 4.21 and 4.22). Exposure to flutamide did not affect stromal vimentin expression but altered the distribution of vimentin in the epithelium. In WDs from fetuses exposed to flutamide *in utero*, vimentin 'spiking' was apparent in the caput of many WDs at e19.5 and e21.5, as well as the cauda, and this spiking became more intense than in control WDs (Fig. 4.22 and 4.23). No significant quantitative difference was seen in protein expression in WDs from flutamide-exposed animals compared to age matched controls (Fig. 4.24).

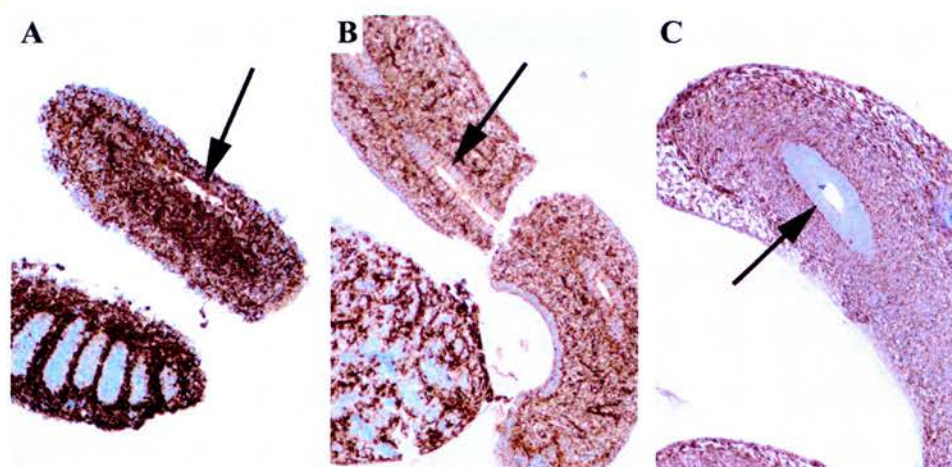


Figure 4.21 *Immunoexpression of vimentin in the caput region of control Wolffian ducts (WD) at e15.5-e19.5.* Note that vimentin (immunostained brown) is expressed in the stroma at all ages but is only expressed in the epithelium (arrow) at e15.5 (A) and e17.5 (B), not at e19.5 (C). Scale bar = 100 μm

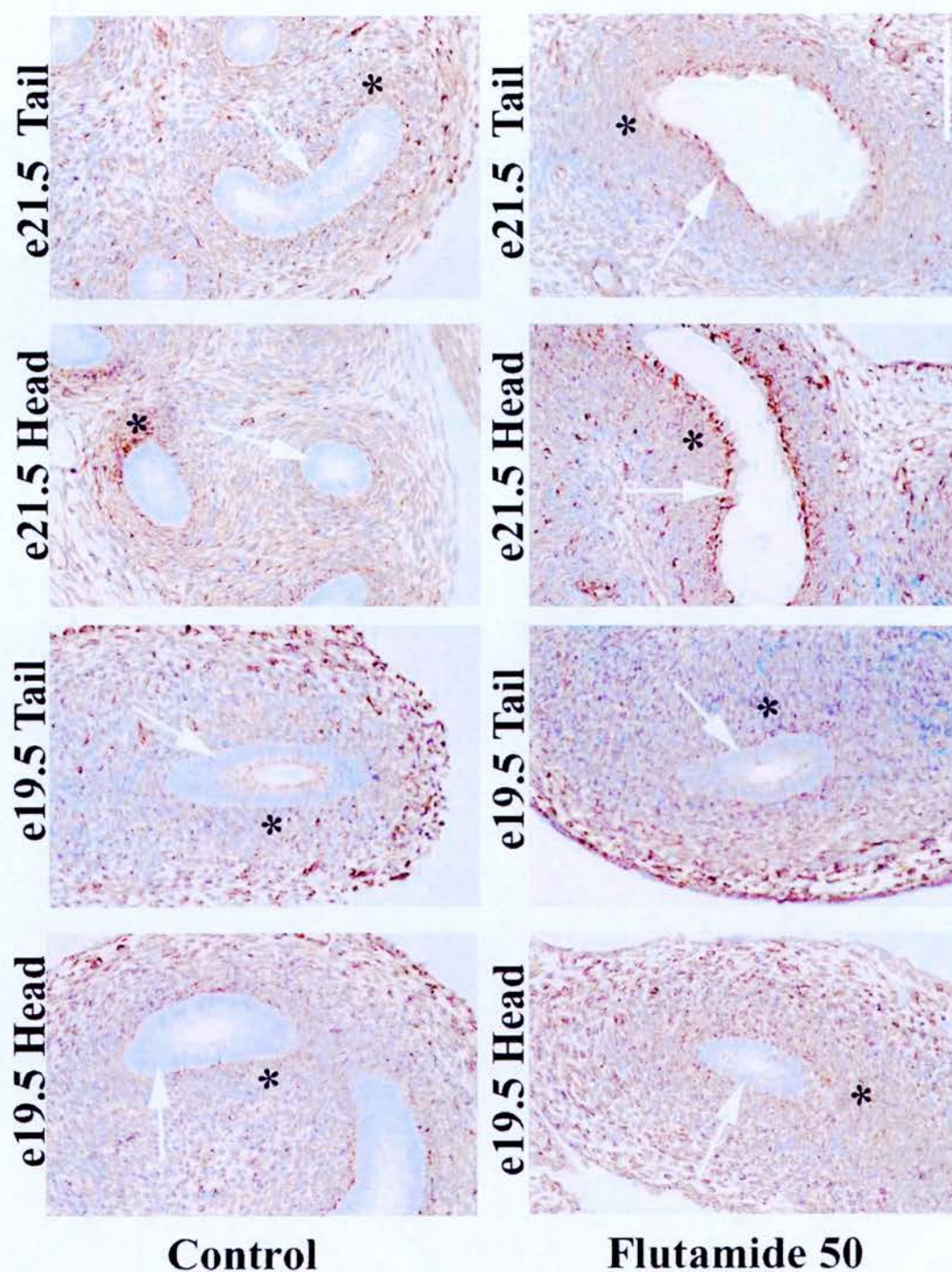


Figure 4.22 Immunoeexpression of vimentin in control (left panel) and flutamide-exposed (50 mgkg^{-1} , right panel) WD at e19.5 and e21.5. Note that vimentin is expressed in the stroma and did not vary with age or flutamide treatment (*, brown). Vimentin was also detected in the epithelium (arrow) but only in the tail region of the epididymis and not in the head region in control animals. Flutamide-exposure increased the prevalence of this

vimentin 'spiking' in the epithelium and it became apparent in the head as well as in the cauda. Scale bar = 100 μ m

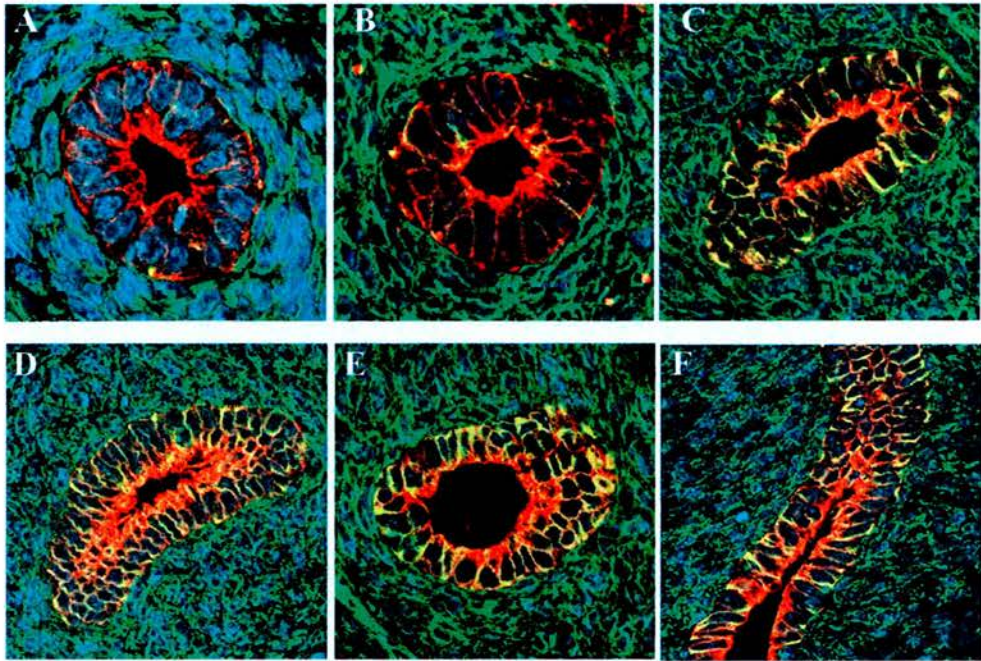


Figure 4.23 Co-expression of vimentin (green) and cytokeratin (red) in e21.5 Wolffian ducts (WDs) from control (A and D) and flutamide-exposed (50 mg/kg-1 B and E, 100 mg/kg-1 C and F) animals. Note that cytokeratin stains the epithelial cell boundaries red. Note that vimentin (green) is not detected in the epithelium in the caput region of the control WD (A) but is seen to co-express with cytokeratin in the cauda (yellow, D). This pattern is altered in flutamide-exposed WDs with epithelial vimentin expression detected in both the caput (B and C) and the cauda (E and F).

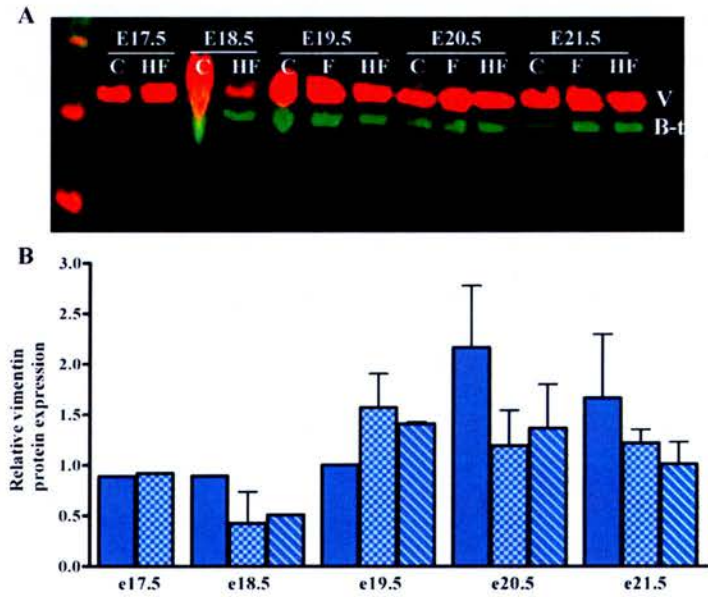


Figure 4.24 Comparative expression of vimentin protein at e17.5–21.5 in Wolffian ducts (WDs) from controls and animals exposed in utero to 50 mgkg⁻¹ or 100 mgkg⁻¹ flutamide. Panel A shows a representative Western blot for vimentin protein (red) in WDs from control (C) and flutamide exposed animals (F - 50 mgkg⁻¹; HF - 100 mgkg⁻¹) at e17.5–e21.5 (n = a pool of 3 WDs per age/treatment, from at least 3 different litters). Panel B shows quantitative analysis of vimentin expression, relative to e19.5 control levels and corrected for loading using β -tubulin (green band, B-t). Note that no consistent change was noted in vimentin protein expression after flutamide-exposure (50 mgkg⁻¹ chequered bars; 100 mgkg⁻¹ striped bars), compared to controls. Values are means \pm S.E.M.

4.3.4 Integrity of the basement membrane in the WD after exposure to flutamide

4.3.4.1 Laminin

Laminin was immunolocalised to the basement membrane in WDs at all ages studied (e18.5–e21.5), forming a ‘ring’ between the epithelium and the stromal compartment (Fig. 4.25). In WDs from flutamide exposed fetuses, the laminin ‘ring’ was interrupted in places and was less defined (Fig. 4.25). In some WDs, the epithelial cells appeared to be sitting in the laminin rather than sitting on the basement membrane.

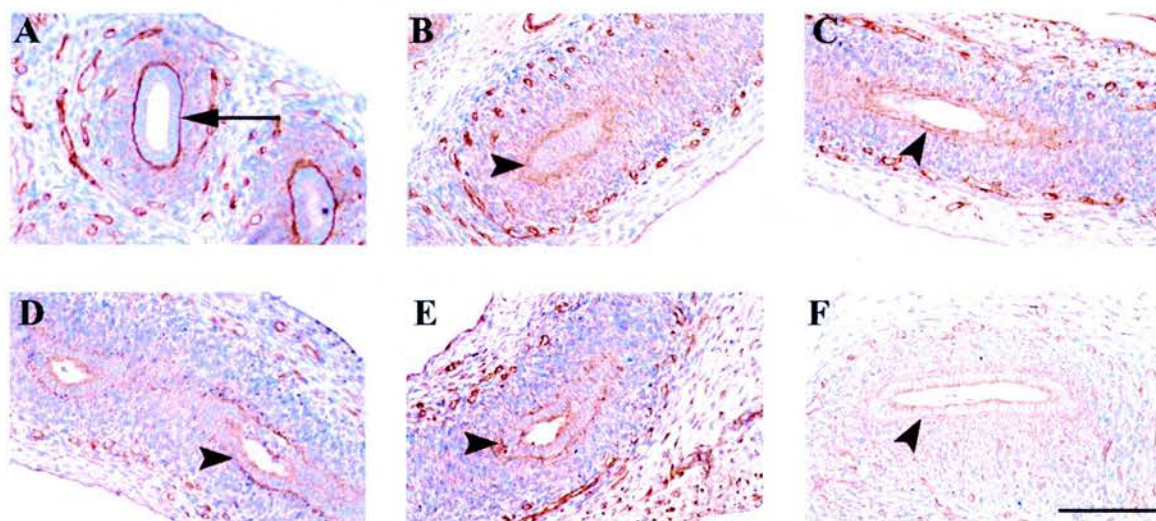


Figure 4.25 Immunoexpression of laminin in the basement membrane of control (A) and flutamide exposed (B-F) WDs at e21.5. Note the defined ‘ring’ of laminin around the base of the epithelium in the control WD (arrow, A). This ‘ring’ became less defined and interrupted in WDs from flutamide-exposed litters (arrowhead, B-F). Scale bar = 100 μ m

4.3.4.2 Fibronectin

Fibronectin is expressed around the basement membrane and the inner stroma of the WD at all ages studied (e18.5–e21.5) with more intense immunoexpression in the cauda than the

caput (Fig. 4.26). WDs from fetuses exposed to flutamide *in utero* showed an increase in fibronectin expression, especially in areas where the epithelium looked abnormal (Fig. 4.27).

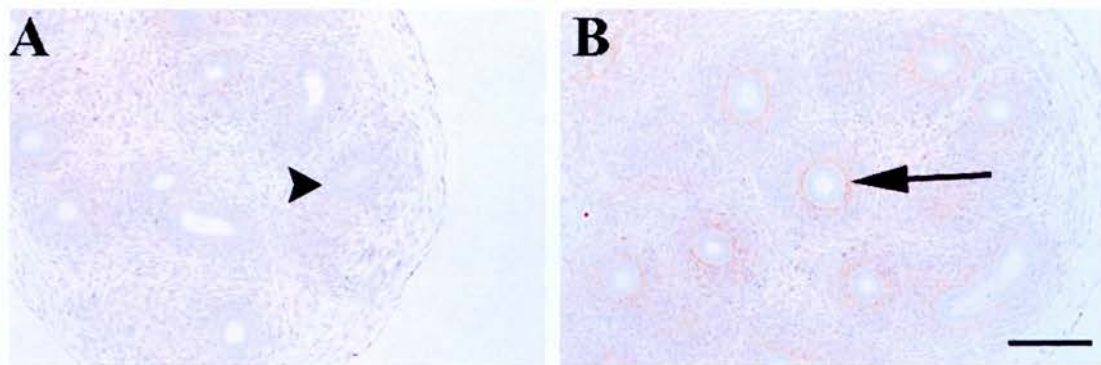


Figure 4.26 *Immunoeexpression of fibronectin (brown) in an e21.5 control Wolffian duct. Note that the staining is more intense in the cauda region (arrow, B) than the caput (arrowhead, A) in the same WD. Scale bar = 100 μ m*

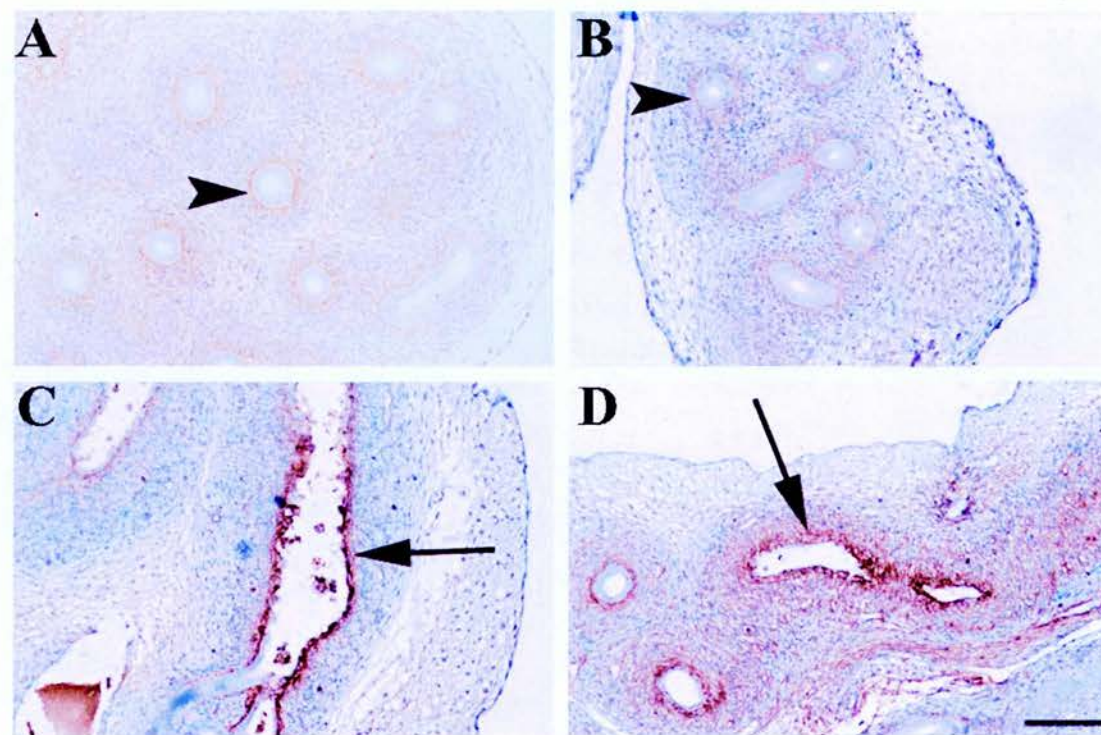


Figure 4.27 *Immunoeexpression of fibronectin in control and flutamide-exposed WDs at e21.5. Note that fibronectin is more intensely expressed in the flutamide-exposed WDs, especially around abnormal epithelia (brown, arrow, C – 50 mgkg^{-1} and D – 100 mgkg^{-1}) than in age matched controls (arrowhead, A and B). Scale bar = 100 μ m*

4.3.5 Degradation of the ECM

4.3.5.1 The role for MMP-2 in the degradation of flutamide-exposed WDs

MMP-2 was immunolocalised to the WD stroma immediately adjacent to the epithelium (Fig 4.28), but no significant difference was seen in immunoexpression or mRNA levels in flutamide-exposed WDs compared to age matched controls (Fig. 4.29). However, this represents both active and inactive forms of MMP-2 and specific analysis of active MMP-2 alone may provide more insight into the role for MMP-2 in the disruption of normal WD development after exposure to flutamide *in utero*.

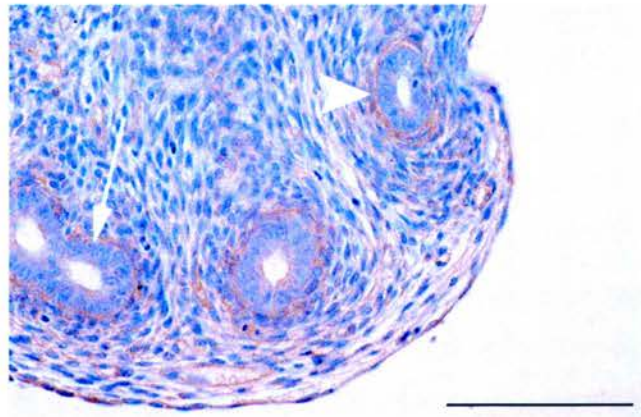


Figure 4.28 *Immunoexpression of MMP-2 in an e21.5 control Wolffian duct. Note that MMP-2 is expressed in the stroma (arrowhead) immediately adjacent to the epithelium (arrow). Scale bar = 100 μ m*

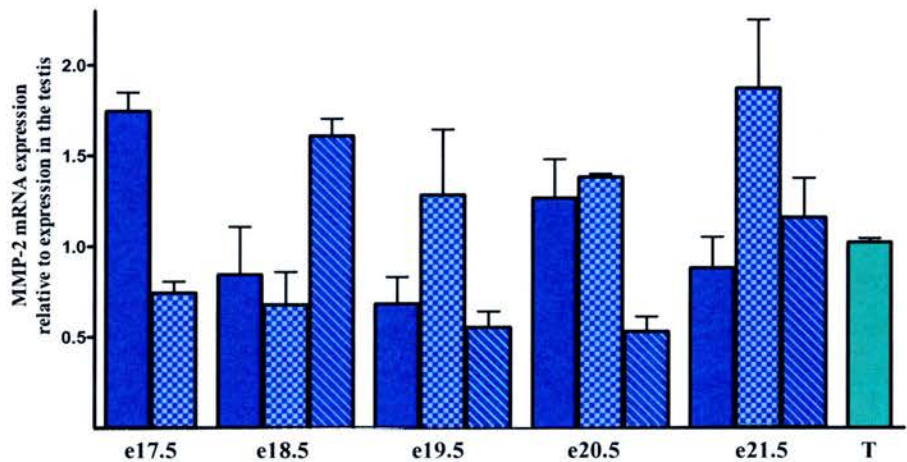


Figure 4.29 *Quantitative analysis of MMP-2 mRNA levels in Wolffian ducts at e17.5-e21.5 in control (solid blue bars) compared to flutamide-exposed animals (50 mgkg^{-1} chequered bars; 100 mgkg^{-1} striped bars). Note that no consistent or significant difference was seen in MMP-2 mRNA expression with age/treatment, as determined by Taqman QRT-PCR (means \pm S.E.M, $n=3-5$ WDs per age/treatment, each from different*

litters). *MMP-2* mRNA expression was expressed relative to mRNA levels in control e21.5 testes (green bar, T) and compared to the internal positive control gene expression.

4.3.5.2 Is the postnatal loss of WD segments and epithelium in flutamide-exposed animals due to an immunological response?

Markers for macrophages (CD68), leukocytes (CD45) and neutrophils (neutrophil elastase) were analysed in the postnatal epididymis by immunohistochemistry. No obvious influx of leukocytes including macrophages or neutrophils was noted in either the control epididymis or the flutamide-exposed epididymis at pnd17-pnd70 (data not shown).

4.3.6 The role for cell adhesion in WD development and its interruption by flutamide-exposure

4.3.6.1 E-cadherin expression

E-cadherin was immunolocalised to the cell boundaries of the epithelium lining the WD at all ages studied (Fig 4.30). Epithelia in flutamide-exposed WDs continued to express E-cadherin protein in the same pattern (Fig 4.30). Quantification of this protein expression highlighted no consistent or significant change in E-cadherin protein in WDs from flutamide-exposed animals (Fig. 4.31).

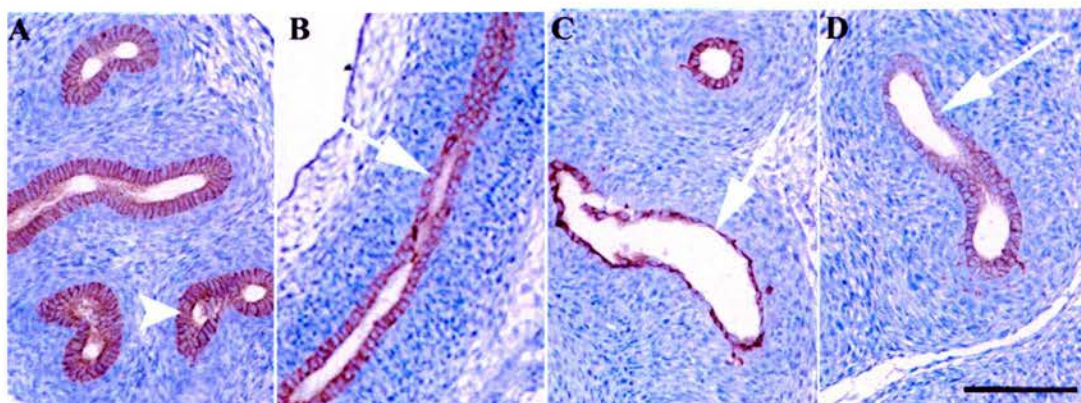


Figure 4.30 *Immunoexpression of E-cadherin in the epithelium of control (A) and flutamide-exposed (100 mg kg^{-1} , B-D) Wolffian ducts (WD) at e21.5. Note that E-cadherin is expressed at the epithelial cell boundary in both control (brown, arrowhead, A) and flutamide-exposed WDs, even when the epithelium is damaged and flattened (arrow, B-D).*

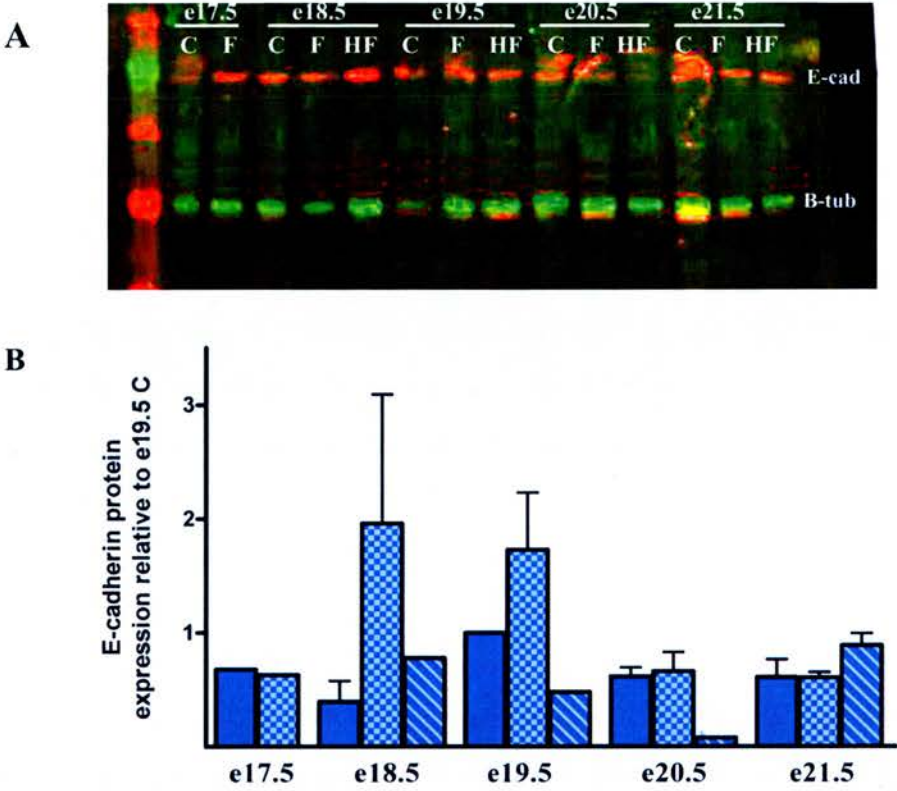


Figure 4.31 E-cadherin protein expression in control and flutamide-exposed Wolffian ducts at e18.5-e21.5. Panel A shows a representative Western blot for E-cadherin (C – control; F – 50 mgkg⁻¹ flutamide; HF – 100 mgkg⁻¹ flutamide). Panel B shows quantification of E-cadherin protein levels in the Wolffian duct (WD) at e17.5 – e21.5 (mean ± SEM) highlighting variation but no significant change in E-cadherin protein expression in WDs from control (solid blue bars) compared to flutamide-exposed animals (checked bars - mgkg⁻¹; striped bars – mgkg⁻¹). n=2 Western blots, each using a pool of 3 WDs per age/treatment, from at least 3 litters. Protein expression was corrected for loading and expressed relative to levels in e19.5 control WD. * p<0.05 compared to age matched control.

4.3.6.2 N-cadherin

N-cadherin was immunolocalised to the inner stromal compartment of an e21.5 WD, immediately adjacent to the epithelium (Fig. 4.32). N-cadherin protein was weakly expressed in WDs from control and low dose flutamide (50 mgkg⁻¹) animals but was more intensely immunoexpressed in WDs from high dose flutamide (100 mgkg⁻¹) animals at e21.5 (Fig. 4.32).

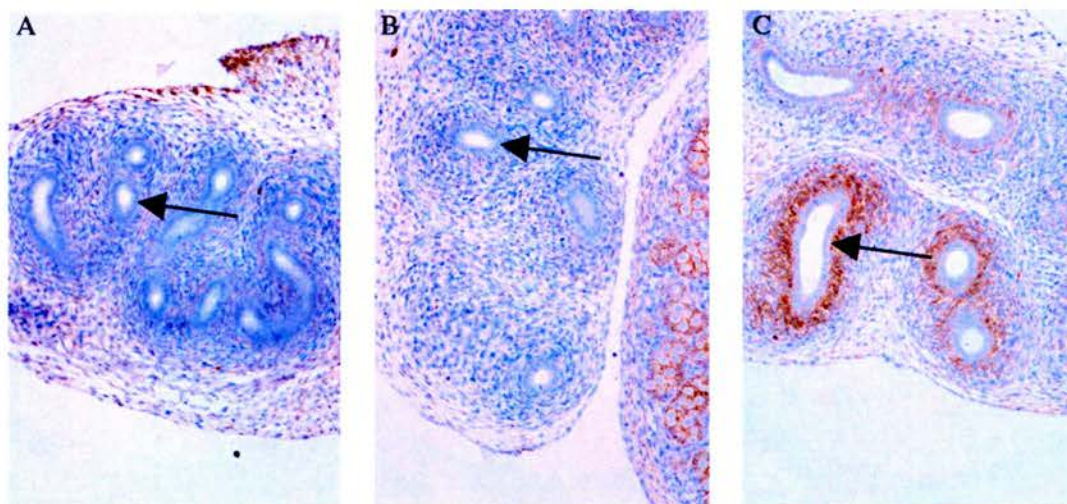


Figure 4.32 Immunolocalisation of N-cadherin to the stroma immediately adjacent to the epithelium in the WD at e21.5. Note that N-cadherin is weakly immunoexpressed in the WD from control (A) and low dose flutamide (B, 50 mgkg⁻¹) animals while N-cadherin is more intensely expressed in the WDs exposed to high dose flutamide (C, 100 mgkg⁻¹). Note that N-cadherin was not detected in the epithelium (arrow).

4.3.7 Hox gene expression in the fetal Wolffian duct

4.3.7.1 Hox A10

Hox A10 mRNA was detected in the fetal WD (e18.5-e20.5, Fig. 4.33) but its localisation and protein expression could not be successfully determined due to technical difficulties with antibodies and *in situ* hybridisation probes. There was an apparent increase in Hox A10 mRNA expression with age, but this was not quantified (Fig. 4.33).

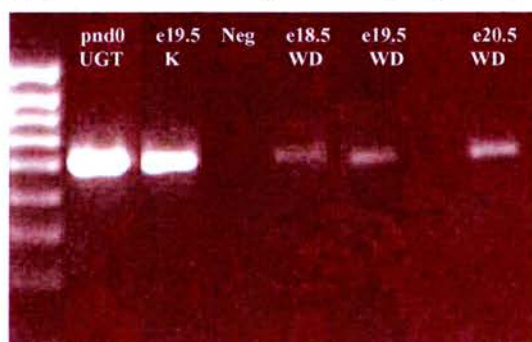


Figure 4.33 Hox A10 mRNA was detected in rat fetal Wolffian duct (WD) at e18.5-e20.5. This expression was low in comparison to expression in the kidney (K) or urogenital tract as a whole (UGT) but there was an apparent, increase in WD expression with age.

4.4 Discussion

It was clear that gestational exposure to flutamide resulted in altered WD development at both the gross and histological level (see chapter 3) but it was unknown what effect this had at the molecular level or what was regulating the (mainly postnatal) loss of segments of the WD.

It has been suggested that the pattern of androgen receptor (AR) expression reflects the androgen responsiveness of the tissue (Bentvelsen *et al*, 1995). AR was mapped to the epithelium from e17.5 and the stroma of the WD from e16.5, however epithelial AR expression was less intense than in stromal cells, particularly before e20.5. This is in agreement with other studies using antibodies demonstrating that ARs are expressed in the nuclei of both the stroma and epithelial cells of the rat WD by e17.5 (Bentvelsen *et al*, 1995; Majdic *et al*, 1995). However, studies in mice using ³H-dihydrotestosterone steroid autoradiography reported stromal expression from e13 but epithelial expression was not detected until e19, much later than in rats (Cooke *et al*, 1991). This difference in timing of expression may be due to species differences or different methodologies. The more intense and earlier immunoexpression of AR in the stroma compared with the epithelium would be consistent with the view that androgen effects on early WD development occur mainly through stromal AR, and that androgens stimulate the stroma to signal to the epithelium to indirectly control epithelial fate (Cunha *et al*, 1992). Flutamide exposure did not alter either the onset of, or the intensity of, AR expression. Therefore WDs from flutamide-exposed animals retained the potential for AR-mediated androgen action. This conclusion is in contrast to findings by others who were unable to detect AR protein by immunohistochemistry in WDs from e21.5 fetuses exposed to 100 mgkg⁻¹ flutamide (Bentvelsen *et al*, 1995). This difference is unexplained though it could be due to rat strain difference or to the use of different anti-AR antibodies and immunohistochemistry techniques.

AR continues to be expressed in the epithelium and stroma of the postnatal WD-derivatives, in a similar pattern to that of the fetal WD. This highlights that WD-derived structures are still capable of responding to androgens throughout life. As well as the androgen peak seen during fetal life (Faiman, 1981; Fujieda *et al*, 1981; Habert and Picon, 1984), males are exposed to another surge in testosterone levels during puberty (Swerdlow, 1981): this testosterone surge may induce another phase of differentiation in the epididymis and vas deferens, as occurs in the secretory epithelium of the seminal vesicles and prostate which undergo functional differentiation around puberty (Cunha and Donjacour, 1987). Since there was a dramatic increase in the prevalence of missing epididymides and/or vasa deferentia

around the time of puberty in males that were exposed to flutamide *in utero*, it was considered possible that these animals were no longer able to respond to androgens and so did not further differentiate and even lost segments of the epididymis and/or vas deferens. However, AR was still detected in the epididymis and vas deferens from postnatal animals that had been exposed to flutamide *in utero*, even in areas that appeared highly damaged and looked morphologically abnormal. This suggests that flutamide exposure during gestation does not alter the pattern of AR expression in the WD and its adult derivatives and that the adult structures still retain the potential for AR-mediated androgen action. This is consistent with reports by previous researchers who showed that even after linuron exposure, AR was still detected in WD-derived tissue at pnd7 (Turner *et al*, 2003). The mechanism behind the increased loss of reproductive structures during puberty still therefore remains somewhat of a mystery.

Testosterone can be metabolised by 5 α -reductase to form the more potent androgen dihydrotestosterone (DHT), or aromatised to oestradiol (George, 1994) but it is unlikely that either of these hormones play a role in WD development. Oestrogen receptor α (ER α) was not detected in the fetal WD, only in the efferent ducts but oestrogen receptor β (ER β), which is highly expressed in many tissues throughout the body (Saunders, 1998), was detected in the WD stroma and epithelium. However, aromatase could not be detected in the fetal WD (unpublished findings) and oestrogen receptor knock out mice (ERKO) and aromatase KO (ArKO) mice have epididymides present in adulthood (Robertson *et al*, 1999; Hess *et al*, 2000) thus suggesting there is no essential role for oestrogens in fetal WD development. It is also unlikely that DHT is required for fetal WD development as 5 α -reductase protein was not detected at any age studied (e17.5-e21.5). This is in agreement with previous studies which showed that DHT was not detected in the WD until after epididymal differentiation is complete (Wilson and Lasnitzki, 1971). Furthermore, 5 α -reductase deficient patients show normal WD differentiation (Imperato-McGinley and Zhu, 2002) while rats exposed to finasteride, a 5 α -reductase inhibitor, show normal WD development (Imperato-McGinley *et al*, 1992). This confirms that WD development is dependent on testosterone action, rather than DHT or oestrogen.

In the WD, there are 2 main cellular compartments, the stroma and the epithelium. These compartments are obvious in all ages studied (e15.5 – adult) and can be easily identified histologically by the differences in cell morphology (see chapter 3). Flutamide does not alter this compartmentalisation of the WD (discussed in chapter 3). Normal cellular organisation and differentiation of these cellular compartments was assessed by examining expression of cell differentiation markers specific to each compartment, namely smooth muscle actin

(SMA) which demarcates the smooth muscle inner stromal layer, cytokeratin which is specific to epithelial cells and p63 which marks basal epithelial cells. In the normal developing WD, cytokeratin is expressed around the boundary of the epithelial cells from at least e15.5 and continues to be expressed in epithelial cells through to adulthood. Cytokeratin plays a role in controlling cell function, for example, epithelial growth and apoptosis, by modulating signal transduction pathways (Paramio and Jorcano, 2002). Cytokeratin expression is not obviously altered by flutamide exposure, and is even expressed in the flat fibroblast-like epithelial cells in the fetal WD and in the abnormally arranged epithelium in the adult epididymis. This suggests that flutamide does not cause epithelial cells to lose their epithelial identity and function, even when the WD and/or epithelium looks abnormal. This is consistent with previous studies which used cytokeratin as a marker that the epithelial compartment was not altered by exposure to linuron (Turner *et al*, 2003; Bowman *et al*, 2005). It has been suggested that epithelium can potentially regulate patterning in an organ by forming barriers and regulating signal transduction (Shook and Keller, 2003). Furthermore, epithelial sheets provide the organ with mechanical structure and form the first line of defence against mechanical disruption. It is likely that the epithelium will undertake these roles in the developing WD and any flutamide-induced interruptions to the epithelium may interfere with these roles.

The basal epithelial cell marker p63 was not detected in the fetal WD but was expressed in basal epithelial cells by pnd17. The timing and cell specific expression of p63 is vital for normal development and functioning of the epithelium (Atanassova *et al*, 2005). Previous studies have shown that p63 expression switches on in the vas deferens at pnd10 and in the epididymis at pnd14 (Hayashi *et al*, 2004; Atanassova *et al*, 2005) but this time point was not examined in these studies. p63 is believed to be a marker of epithelial differentiation, plays a role in epithelial cell proliferation and, through reciprocal signalling, promotes proliferation and differentiation of the underlying mesenchyme (Levrero *et al*, 2000; Ince *et al*, 2002; Lee and Kimelman, 2002). Previous studies have shown that p63 expression switches on in a caudal-cranial direction with epididymal coiling occurring while p63 levels are low and coiling ceasing once p63 levels reach adult level (Hayashi *et al*, 2004). Expression of p63 was seen to continue throughout postnatal life and was still detected in the epididymides of males exposed to flutamide *in utero* (pnd17-70). However, p63 expression was reduced in flutamide-exposed males with some cross-sections of epididymis having reduced or even lacking p63 staining but this reduction was not quantified. This suggests that the epididymal epithelium does not fully differentiate until postnatal life but that this pattern is established by androgens during fetal life. Furthermore, this reduction in p63

expression may explain the smaller epididymides and reduced cell proliferation in males exposed to gestational flutamide since basal cells are known to influence behaviour of the epithelium (Prins *et al*, 2001). This differs from the urogenital sinus in which p63 staining was seen by pnd0. It is thought that since p63 might regulate the cessation of WD coiling, differences in p63 expression might account for the regional differences in WD morphology (Hayashi *et al*, 2004) thus altered p63 expression might explain the abnormalities seen in flutamide-exposed WDs. Previous studies have reported that mutation in p63 can result in urogenital developmental defects with p63 being essential for normal modelling of female external genitalia (Ince *et al*, 2002). It was not examined if flutamide exposure *in utero* delayed the onset of p63 expression thus highlighting delayed epithelial differentiation: this therefore requires further investigation. Furthermore, in one adult male, the epididymal epithelium appeared folded and stratified, unlike the simple epithelium in controls; this epithelium had an increase in the number of p63 stained cells, which was no longer restricted to the basal cells. This might explain the over-proliferation of the epithelium in this animal as p63 is reported to play a key role in maintaining cell populations (Wang *et al*, 2001) and over proliferation in adult prostates from males exposed neonatally to DES was believed to be due to altered expression of p63 (Risbridger *et al*, 2001).

Unlike epithelial differentiation, smooth muscle stromal differentiation occurred during fetal life with smooth muscle actin (SMA), an androgen responsive stromal differentiation marker (Schlatt *et al*, 1993), detected in the stroma immediately adjacent to the epithelium in the WD corpus by e18.5. It is not understood why SMA is first expressed in the corpus and progresses towards the caput and cauda by e20.5, since the corpus is the segment of the WD which coils last. SMA protein levels were seen to increase with age in control WDs: this agrees with previous studies highlighting an increase in SMA between e17 and e21 (Hannema *et al*, 2006). Blocking androgen action by flutamide exposure impaired stromal differentiation as indicated by reduced expression of SMA protein in the WD. This reduction in smooth muscle differentiation may explain the reduction in WD coiling seen after flutamide exposure (chapter 3). The flutamide-induced reduction in SMA expression was noted as early as e19.5, before any obvious signs of impaired epithelial development were apparent and before the epithelium undergoes differentiation even in the control animals. This is consistent with previous studies in various organ systems which have shown that the stromal cells, via paracrine interactions, play a critical role in controlling epithelial proliferation, differentiation and development (Cunha *et al*, 1985; Donjacour and Cunha, 1991; Cooke *et al*, 1997; Arnold *et al*, 2001; Cunha *et al*, 2002; Marker *et al*, 2003). However, in the seminal vesicles, it has been suggested that the thickness of the smooth

muscle is determined by interactions with glandular epithelium during branching morphogenesis (Marker *et al*, 2003), highlighting possible reciprocal interactions from the epithelium to regulate differentiation of the stroma. The mechanisms underlying these paracrine interactions are poorly understood but growth factors and/or extracellular matrix components are likely to be involved; this therefore warrants further investigation.

The periductal smooth muscle layer persisted in the postnatal epididymis and vas deferens and gestational exposure to flutamide appeared to increase the thickness of this layer. In the vas deferens, a thick SMA negative layer is present between the epithelium and the smooth muscle layer: this is believed to consist of undifferentiated fibroblast-like cells (Atanassova *et al*, 2005). Exposure to flutamide results in an increase in this SMA negative layer. Previous studies have also reported a widening of this layer and have suggested it may be due to altered androgen-oestrogen balance (Chang *et al*, 1999; Atanassova *et al*, 2005). It is also believed that thickening of this periductal fibroblast layer may act as a physical barrier to interfere with normal signalling between the epithelium and stroma (Chang *et al*, 1999).

The extracellular matrix (ECM) surrounding cells is vital not only to provide mechanical support for these cells but it also plays a role in controlling their behaviour and proliferation and differentiation by regulating the availability of signals, such as growth factors, reaching their cell surface (Streuli and Gilmore, 1999). ECM is composed partially of intermediate filaments, such as desmin and vimentin, and previous studies have shown that various components of the ECM increase with age in the developing WD (Hannema *et al*, 2006). Desmin is one of the earliest markers of mesenchymal differentiation into muscle (Alberts, 1994) and was detected in the WD stroma at all ages studied (e15.5-e21.5). This again highlights the early differentiation of the WD stroma. Desmin is still expressed in flutamide-exposed WD stroma, showing no consistent change compared to age matched controls. Intermediate filaments are required not only to support cells but are also involved in signal transduction from the cell membrane to the nucleus; desmin may therefore play a role in signal transduction in the developing WD.

Vimentin, an early mesenchymal intermediate filament, was also expressed in the fetal WD. It was constitutively expressed in the WD stroma at all ages studied (e18.5-e21.5) and this was not obviously affected by flutamide-exposure. Vimentin is likely to be providing mechanical support to the developing WD as well as possibly mediating signalling through interactions with and organisation of the cytoskeleton (Alberts, 1994). Vimentin was also expressed on the basolateral boundary of the epithelial cells: in control WDs this 'spiking' was seen along the full length of the WD between e15.5-18.5 but becomes restricted to the caudal region by e19.5. It is thought that vimentin expression often precedes expression of

other intermediate filaments during differentiation, for example desmin. Since vimentin is initially expressed in the epithelium along the full length of the WD at e15.5 and becomes later restricted mainly to the cauda in control WDs by e19.5, it is likely that vimentin expression in the epithelium is replaced by another intermediate filament in a cranio-caudal direction as development proceeds. This hypothesis is substantiated by evidence from studies in 8 week human fetuses in which both the MD and the WD co-express vimentin and cytokeratin at the apical and basolateral edges of the epithelium while the surrounding mesenchyme only expresses vimentin, not cytokeratin (Magro and Grasso, 1995). Epithelial 'spiking' of vimentin was detected along the full length of the epididymal segment of the flutamide-exposed WD, rather than just in the cauda at e21.5. This could be due to failure of the epithelial cells to differentiate and so fail to switch off epithelial vimentin expression and thus epithelial cells retaining some mesenchymal-like properties. This might explain the reduced epithelial cell height and loss of attachment to the basement membrane seen in flutamide-exposed WDs. Vimentin is required for cell motility and increased vimentin expression is seen in prostate cancer cells as they become more invasive (Singh *et al*, 2003). It could therefore be hypothesised that these vimentin positive WD epithelial cells may be gaining motility but there is no definitive evidence for this, and further investigation of earlier and later time points might provide some insight. Studies in the equine endometrium have shown that in cases of endometriosis, cytokeratin and vimentin become co-expressed in the epithelium, which is hypothesised to be due to increased proliferation and a loss of cell to cell contact (Aupperle *et al*, 2004). Other researchers have also shown that altered hormone action could induce epithelial vimentin expression (Klymkowsky, 1989). These hypotheses might explain the atypical expression of vimentin in flutamide-exposed WD epithelium.

The basement membrane, a thin tough sheet of ECM, plays an important role in mediating epithelial cell adhesion, gene expression, proliferation and differentiation (Danen and Yamada, 2001). Laminin was expressed in the WD basement membrane as a clearly defined 'ring' around the base of the ductal epithelium. Flutamide exposure however interrupted this laminin 'ring' resulting in a less defined and discontinuous basement membrane. Laminin anchors epithelial cell surfaces to the basement membrane and epithelial cells must attach to the appropriate ECM component in order to survive since loss of contact with specific ECM components is recognised as a pro-apoptotic signal (Frisch and Francis, 1994; Danen and Yamada, 2001). Therefore interruption to the basement membrane could result in epithelial cells losing their attachment and so sloughing off or losing their polarity and epithelial identity: previous studies have also highlighted this loss of basement membrane in the regressing WD, resulting in possible mesenchymal transformation (Jirsova

and Vernerova, 1993). It has also been suggested in the MD that epithelial cells can protrude through breaks in the basement membrane and are phagocytosed, allowing neighbouring cells to de-differentiate into mesenchymal cells and migrate off (Trelstad *et al*, 1982; Austin, 1995; Zhan *et al*, 2006). This might explain the loss of epithelia in sections of the WD and the appearance of flattened mesenchymal-like cells (see chapter 3). This phenomenon is apparent in the regression of the MD whereby the basement membrane becomes interrupted and detached with apoptosis only evident in the mesenchyme after the basement membrane has become discontinuous (Magro and Grasso, 1995); (Allard *et al*, 2000). It is believed that the loss of cell contact with specific ECM components acts as a pro-apoptotic signal resulting in regression of the MD (Allard *et al*, 2000).

Fibronectin accumulates along the interface between epithelium and mesenchyme and attaches cells to a variety of extracellular matrices, except type IV matrices that involve laminin as the adhesive molecule (Ruoslahti, 1981). Like many other cytoskeletal proteins, fibronectin plays a role in regulating cell structure and behaviour and redistribution of fibronectin can be associated with epithelial differentiation (Paranko *et al*, 1983). Fibronectin was expressed in the inner stroma as a less defined 'ring' just below the basement membrane in the fetal WD and is likely to be involved in regulating the attachment of stromal cells to the surrounding ECM and mediating cell signalling. This expression was more intense in the caudal segment of the future epididymis than in the caput: it is unclear why there would be more fibronectin in the cauda. Fibronectin synthesis is an early sign of smooth muscle differentiation so this increased expression in the cauda might represent stromal differentiation (Paranko *et al*, 1984). Fibronectin expression was increased in flutamide- exposed WDs, especially in areas where the epithelium looked abnormal and flattened. This suggests increased adhesion of stromal cells to the surrounding ECM but it remains something of a mystery why flutamide would induce this and the resulting effect on WD morphology and function. This finding is in contrast to reports by Turner and colleagues who saw no change in fibronectin expression in WDs after gestational exposure to linuron (Turner *et al*, 2003). Our findings also contradict reports of reduced fibronectin synthesis and discontinuous periductal expression seen during MD regression (Paranko *et al*, 1984). Like epithelial cells, fibroblasts must adhere to the correct ECM in order to survive and function normally (Danen and Yamada, 2001), therefore altered attachment to the ECM could have a negative effect on stromal integrity and function. Changes in fibronectin levels have been associated with altered epithelial cell proliferation in response to hormones (Woodward *et al*, 2001), therefore flutamide-induced altered fibronectin expression may play a role in reducing epithelial cell proliferation. Furthermore, fibronectin

has been reported to increase MMP-2 activity and induce epithelial cell loss in hormone withdrawal- induced mammary gland involution (Schedin *et al*, 2000). Therefore it is possible that the increase in fibronectin in flutamide-exposed animals might play some role in the loss of epithelium and entire segments of the WD.

E-cadherin was expressed around the boundary of epithelial cells in the WD and exposure to flutamide did not consistently alter this expression whereas N-cadherin was expressed in the inner stromal compartment and appeared to be more intensely expressed in WDs exposed to high (100 mgkg⁻¹), but not low doses of flutamide (50 mgkg⁻¹). This increase may not reflect a true increase in N-cadherin protein expression as it has been suggested that as N-cadherin dissociates from the cell, it is more easily immunodetected. This may be true for WDs exposed to 100 mgkg⁻¹ flutamide but would require further investigation by Western blot analysis. Cadherins not only act as 'biological glue' but they also play a role in generating signals at the cell surface that can influence cell function and appropriate expression of cadherins is vital to maintain tissue integrity (Rowlands *et al*, 2000). It has even been suggested that the manner in which cells respond to hormones is dependent on adhesive interactions with other cells (Byers, 1993). It is possible therefore that E-cadherin and N-cadherin are playing a role in maintaining normal tissue integrity in the WD and in controlling how cells respond to androgens.

Taken together these results suggest flutamide-exposure can disrupt the formation and maintenance of the cytoskeleton, intermediate filaments and basement membrane: this might provide some explanation for the morphological effects of flutamide on the developing WD (see chapter 3). It is unclear what is causing this disruption to the ECM and WD degradation however it was hypothesised that MMPs may play a role since they are well known for degrading the components of the ECM (Alberts, 1994). Analysis of MD regression in males has shown that degradation of the ECM is evident and that MMP-2 plays a role downstream of paracrine signalling (Roberts *et al*, 2002), thus it would be unsurprising if MMPs also played a role in flutamide-induced WD abnormalities. Consistent with this, MMP-2 protein was expressed in the inner stromal compartment of the WD but there was no consistent trend in changes in MMP-2 mRNA expression in flutamide-exposed WDs compared to age matched controls. This represents both inactive and active forms of MMP-2, therefore further analysis is required using zymography to gain insight into this. This hypothesised role for MMPs in WD abnormalities is further substantiated by data arising from gene array studies examining changes in gene expression in control compared to linuron-exposed rat WDs at pnd7. Linuron induced a decrease in TIMP3, an MMP inhibitor. TIMP3 is localised to the epithelium and binds to the ECM thus locally regulating MMP activity in the ECM

(Turner *et al*, 2003). Furthermore, Bowman and colleagues reported a trend for increased MMP gene expression in the fetal WD after gestational exposure to DBP (Bowman *et al*, 2005). Taken together, these results suggest that MMPs might contribute to the abnormalities seen in WD development. Previous studies in the endometrium have reported that MMP-2 is synthesised by the stromal compartments but it requires a soluble epithelial factor to be activated (Goffin *et al*, 2002). This might be true also in the WD and flutamide-induced epithelial abnormalities may activate MMP-2 to degrade WD tissue. Alternatively, it could be hypothesised that the disruption to the ECM and the subsequent loss of segments of the WD may be due to an influx of immune cells. However, there was no obvious immunological response seen in either flutamide-exposed fetal WDs or in the adult-derivatives. This is in contrast to findings published showing that neonatal exposure to DES can result in an influx of immune cells into the postnatal vas deferens with deleterious consequences (Atanassova *et al*, 2005).

Retinoid X receptor alpha (RXR α) is a member of the retinoic acid receptor family and functions as a ligand-dependent transcription factor. RXRs are expressed in a spatially restricted pattern in the developing embryo and respond to high concentrations of RA. RXRs can form dimers with the AR and, in the presence of ligand RXR can suppress AR target genes while unliganded RXR can enhance AR transactivation (Chuang, 2005). Similarly, AR can suppress RXR target genes thus preventing RXR binding to the response element in target genes (Chuang, 2005). Other studies on going in the lab had shown changes in RXR expression in the testis after exposure to anti-androgens therefore RXR expression was examined in the WD. RXR α is expressed in the stroma and epithelium of the WD at all ages studied (e17.5-21.5) and immunoexpression appeared reduced in flutamide-exposed WDs. RXR α may play a role in regulating AR target gene expression in the WD thus contributing to the normal development of the WD: the flutamide-induced reduction in AR signalling may cause this decrease in RXR α expression. Further investigation is required to interrogate fully the role for retinoid and their receptors in WD development.

Hox genes are known to play a role in fetal development, particularly in patterning and segmentation (Favier and Dolle, 1997). It has been suggested that sex steroid hormones might act to establish Hox gene expression (Block *et al*, 2000), for example, in the uterus (Taylor *et al*, 1998; Taylor *et al*, 1999). Reproductive tracts in both males and females are an example of developmental axes thought to be controlled by Hox genes. Notably, Hox genes A9, A10, A11 and A13 are reported to be expressed along the length of Wolffian and MDs with their expression persisting into adulthood (Podlasek *et al*, 1999; Block *et al*, 2000; Bomgardner *et al*, 2001). Hox A11 is reported to be expressed in the mesenchymal cells of

the developing Wolffian duct and in the stroma of the adult vas deferens but not in the epithelial lining (Hsieh-Li *et al*, 1995) and Hox A11 homozygous mutants are sterile with males presenting with small, abnormally coiled vasa deferentia (Hsieh-Li *et al*, 1995). Furthermore, mice homozygous for targeted deletion of Hox A10 exhibit abnormal coiling of the cranial portion of the vas deferens, again highlighting the role for Hox A10 in Wolffian duct development (Benson *et al*, 1995; Podlasek *et al*, 1999). These studies suggest that Hox A10 expression may delineate the boundary between the epididymis and vas deferens, and that targeted deletion blurs the normal epididymis-vas boundary (Podlasek *et al*, 1999). Current studies have shown that Hox A10 mRNA is expressed in the developing rat WD from e17.5; however, technical difficulties have prevented any further progress in our understanding of the role for Hox genes in the rat WD.

4.5 Conclusion

During WD development, AR expression is switched on early in the stroma then later in the epithelium, around the time of morphological differentiation. This suggests that early androgen action is mediated by the stromal cells. Flutamide-exposure did not alter AR expression indicating that these WDs are still capable of AR mediated androgen action. The fetal WD is divided into an epithelial and a stromal compartment in both control and flutamide-exposed animals, however the differentiation of these cells types is altered by flutamide-exposure. This is first evident in the stroma during fetal life and is not observed widely or consistently in the epithelial compartment until postnatal life. Together this evidence is consistent with the view that during male reproductive development, androgens act on the stroma which in turn mediates epithelial development. Furthermore this evidence suggests that the patterning of WD compartments and differentiation is established during fetal life, even if abnormalities do not become apparent until adulthood.

The ECM is vital to provide cells with mechanical support as well as playing a role in mediating cell signalling. Various extracellular matrices are present in the developing WD including laminin, fibronectin and vimentin and exposure to flutamide interferes with their normal expression pattern: the mechanisms involved in this ECM degradation are not yet fully understood but MMPs may contribute. The flutamide- induced loss of an intact basement membrane and ECM may be responsible for the loss of segments of the WD, rather than the action of immune cells. Future studies will focus on understanding the timing of androgen action in WD development as well as further investigating the mechanisms behind androgen dependent WD development.

5 Determination of the key time window of androgen action necessary for WD development

5.1 Introduction

The results presented in chapters 3 and 4 confirmed the vital need for androgen action in WD development, with WD differentiation apparently being more susceptible to reduced androgen action than is stabilisation. These studies and those previously reported in the literature have shown that androgens are necessary to mediate both stabilisation and differentiation of the WD but these have not determined the actual window of androgen action in WD development (Imperato-McGinley *et al*, 1992; Foster *et al*, 2001; McIntyre *et al*, 2002). It is generally presumed that androgen action is required during the window of morphological differentiation, however this remains to be confirmed: the studies detailed in this chapter were designed to answer this question and to establish the key window(s) of androgen action in the developing WD.

One previous study has examined the effects of exposure to anti-androgens for short defined periods during male reproductive development and compared this with exposure throughout male reproductive development. Wolf and colleagues exposed pregnant rats to vinclozolin for various 2 day windows during the period of fetal reproductive development and noted that extended exposure to vinclozolin (400 mg kg^{-1}) between e14-19 resulted in more severe, and an increased prevalence of, reproductive tract abnormalities than did exposure for just 2 days (Wolf *et al*, 2000). They reported that different structures in the reproductive tract have different windows of sensitivity. For example, AGD was significantly reduced in males exposed to vinclozolin between e16-17 and e18-19 but not in males exposed to vinclozolin earlier in fetal life (Wolf *et al*, 2000). The most sensitive period for exposure to vinclozolin appeared to be between e16-17, which resulted in abnormalities mainly in the external genitalia, suggesting this to be the window of androgen action in external genitalia development (Wolf *et al*, 2000). However, these studies did not provide insight into the window of androgen action in WD development, as exposure to vinclozolin for just 2 days between e12-13, 14-15, 16-17, 18-19 or 20-21 did not result in any obvious abnormalities in WD-derived structures in adulthood (Wolf *et al*, 2000). This is not surprising since in a previous study Gray and colleagues (1994) saw no epididymal defects in rats exposed to vinclozolin (200 mg kg^{-1}) between e14-pnd3 suggesting that vinclozolin is insufficient at blocking androgen action in the developing WD.

Another such study examining the effects of short term exposure to anti-androgens has been undertaken in the mouse (Silversides *et al*, 1995). This study showed that exposure to

hydroxyflutamide (30 mg) on e11-15 resulted in smaller epididymides and infertility whereas exposure on e15-20 prevented normal prostate development: exposure to hydroxyflutamide on e19-20 did not result in any obvious reproductive abnormalities or infertility in adulthood (Silversides *et al*, 1995). These studies suggest that high levels of androgens may not be essential later in WD development, during its morphological differentiation, but none of the dosing regimes used by these researchers induced such gross abnormalities as those seen in the studies presented in this thesis. This may be due to the use of hydroxyflutamide by Silversides and colleagues therefore these studies are not directly comparable to those in this thesis. Again, these studies do not address the precise window of androgen action in WD development and insight into this may provide further understanding of the mechanisms of androgen action, not just in the WD but in the entire reproductive tract.

5.1.1 Aims

To investigate the critical window of androgen action necessary for WD development.

To establish if androgens act during the period of morphological differentiation and/or earlier in development.

5.2 Methods

5.2.1 *In utero* treatments

As detailed in section 2.2, pregnant dams were randomly allocated to treatment groups and dosed once daily by gavage with 1 ml kg⁻¹ maternal bodyweight of the following treatments. Dams were dosed with 100 mg kg⁻¹ flutamide either from the onset of androgen production by the testis until birth (e15.5-21.5) or during critical windows to encompass the period of WD stabilisation (e15.5-17.5, early exposure) or the period of WD morphological differentiation (e19.5-21.5, late exposure) (see Fig. 5.1).

Control: 1ml corn oil/2.5% DMSO (n=10 litters)

Flutamide: 100mg kg⁻¹ in 1ml corn oil/2.5% DMSO

e15.5-e21.5 - n=11 litters

e15.5-e17.5 - n=5 litters

e19.5-e21.5 - n=6 litters

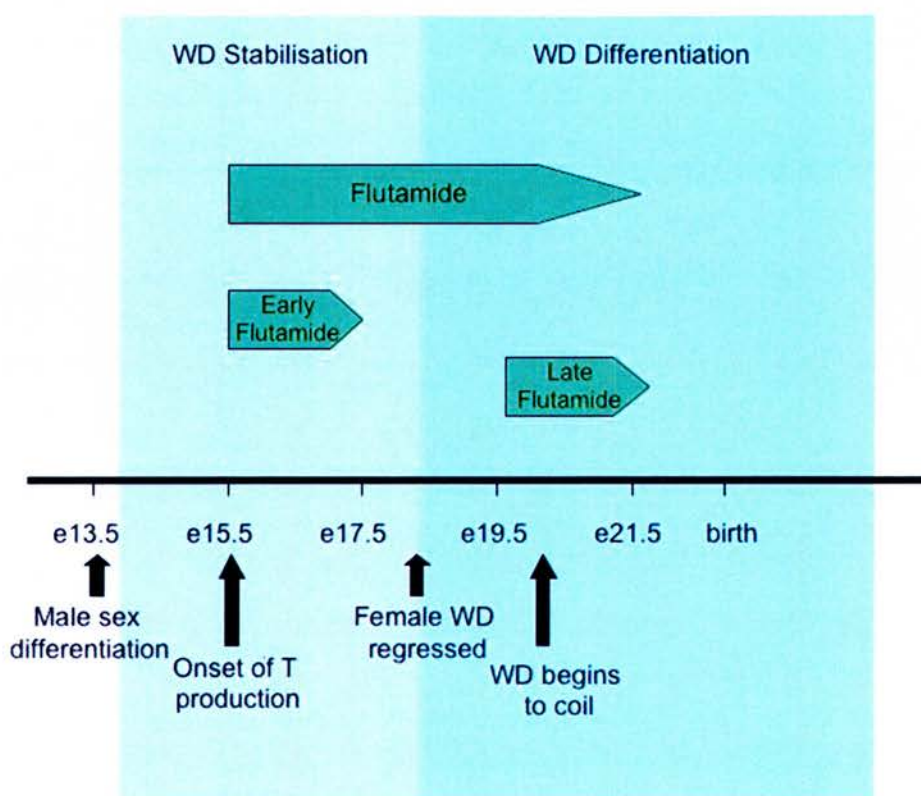


Figure 5.1 Summary of windows of flutamide exposure used

This dose of flutamide was selected based on results previously reported in chapters 3 and 4 highlighting a high incidence of reproductive tract abnormalities.

Throughout this chapter, offspring from dams treated with flutamide while pregnant will be referred to as “flutamide-exposed” or treated “*in utero*”. This does not mean that fetuses were directly exposed to flutamide but were exposed indirectly via their mother.

5.2.2 Tissue collection

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation and fetuses were recovered at e18.5 and e21.5. Postnatal animals, older than pnd10, were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Fetuses (e21.5) and postnatal animals were weighed and anogenital distance measured as described in section 2.3.

Fetuses were killed by decapitation before reproductive tracts were recovered by microdissection and photographed using a Leica ICA camera (section 2.3.2.1). Reproductive tracts and gonads were collected from postnatal animals and photographed as described in section 2.3.2.2. Reproductive tracts were analysed microscopically, at the time of dissection, for any gross morphological abnormalities and tissue was either fixed or frozen as detailed in section 2.4.

5.2.3 Luminal length analysis

Differentiation of fetal WDs was quantified by digitally measuring the luminal length of the epididymal segment of WDs from control and treated animals, as described in section 2.8.

5.2.4 Epithelial height and width measurements

WD sections were immunostained for pan-cytokeratin as detailed in section 2.6.1 to label clearly all epithelial cells. Sections were viewed using the x63 objective on an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Image-Pro Plus version 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) was utilized to measure epithelial cell height and width. Using a x63 objective, epithelial cell height and width was measured in every 5th epithelial cell in the future epididymal segment in a randomly selected section. Height measurements were performed separately for the caput, corpus and cauda regions of the future epididymal portion of the WD. Only epithelial cells in which the nucleus could be

clearly identified were measured, thus excluding from analysis any epithelial cells from the flutamide treatment groups that were severely flattened or disintegrating.

5.2.5 Immunohistochemistry

Immunohistochemistry was performed on isolated reproductive tracts fixed in Bouin's, using standard avidin peroxidase protocols in order to highlight the location of proteins of interest, as detailed in section 2.6.1.

Briefly, sections were cut from paraffin blocks and mounted on glass slides. Sections were then dewaxed and rehydrated then pressure cooked in citrate buffer if required (Table 5.1). Non-specific binding was blocked before adding the appropriate primary antibody overnight, as detailed in Table 5.1. A biotin labelled secondary antibody was used that had been raised against a species-specific sequence on the primary antibody. This biotinylated secondary antibody signal was amplified by incubation with avidin-biotin conjugated with horseradish peroxidase and the signal was localised using DAB. Sections were counterstained with haematoxylin and mounted with glass coverslips. Further detailed protocols can be found in section 2.6.1.

Table 5.1 Summary of primary antibodies used for immunohistochemistry

Target Antigen	Source	Dilution	Retrieval	Host Species
Androgen Receptor	Santa Cruz	1:50	Citrate	Rabbit
Smooth Muscle Actin	Sigma	1:10000	None	Mouse
Pan-cytokeratin	Sigma	1:200	Citrate	Mouse
Vimentin	DAKO	1:1000	Citrate	Mouse
Laminin	Abcam	1:100	Citrate	Rabbit

5.2.6 Western blots

Western blotting was carried out to separate a mixture of proteins according to their molecular size, as detailed in section 2.6.2. Briefly, protein was extracted from frozen e21.5 WDs using RIPA lysis buffer (section 2.6.2.1) and 15ug of each protein was loaded into an SDS-page gel and subjected to electrophoresis at 100 V for 1-2h. Proteins were electro-transferred from the gel and immobilised onto a nitrocellulose membrane. Proteins were subsequently detected on the membranes using specific antibodies (Table 5.2) as described in section 2.6.2.5. An anti- β -tubulin antibody (Sigma) was used as a standardization loading control, diluted 1:300 in TBST.

Table 5.2 Summary of primary antibodies used for Western blotting

Target Antigen	Source	Dilution	Host Species
Androgen Receptor	Santa Cruz	1:300	Rabbit
Smooth Muscle Actin	Sigma	1:10000	Mouse

5.2.7 RNA extraction

RNA was extracted from frozen WDs at e17.5–e21.5 using RNeasy Mini extraction kits (Qiagen, Crawley, UK) and quantified using RNA 6000 Nano chips on an Agilent 2100 Bioanalyser (Agilent Technologies, Wokingham, UK), as detailed in section 2.7.1–2.7.2.

5.2.8 Taqman QRT-PCR

Random hexamer primed cDNA was prepared using the Applied Biosystems TaqMan ® reverse transcription kit (Applied Biosystems, Foster City, CA) (section 2.7.3.2) and quantitative PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Expression of AR mRNA was determined using the Assay-On-Demand Gene Expression ™ system (Rn00560747, Applied Biosystems). Detailed protocols can be found in section 2.7.5.

5.3 Results

5.3.1 Effects of maternal exposure to flutamide during critical windows of reproductive development

5.3.1.1 Non-reproductive effects of flutamide exposure

Consistent with previous studies (McIntyre *et al*, 2001; Foster and Harris, 2005), exposure to flutamide (100 mgkg^{-1}) did not result in any toxicological effects to the dam with no obvious problems with labour, no increase in fetal death or decrease in litter number (data not shown). Furthermore, there was no significant decrease ($p>0.05$) in male bodyweight at e21.5 between control fetuses and those from litters maternally exposed to flutamide (100 mgkg^{-1}) either early (e15.5-17.5) or late (e19.5-21.5) in WD development (data not shown).

5.3.1.2 Effects of flutamide exposure on the reproductive tract

Maternal exposure to flutamide within critical windows of development (100 mgkg^{-1}), as expected, had no obvious effect on fetal female reproductive tract morphology but impacted on normal masculinisation of fetal males. Normally, fetal sex can be determined externally by the distance between external genitalia and the anus (anogenital distance; AGD), with AGD significantly larger in control fetal males than in females (Fig 5.2, $p<0.001$). However, maternal exposure to flutamide during the entire window of reproductive development (e15.5-21.5) prevented normal masculinisation of fetal external genitalia, with AGD significantly reduced ($p<0.001$) at e21.5 to a length similar to that of control females (as discussed in section 3.4.3.2). Exposure to flutamide (100 mgkg^{-1}) early in WD development (e15.5-17.5) reduced AGD by the same extent as exposure to flutamide from e15.5-21.5 whereas maternal exposure to flutamide late in WD development (e19.5-21.5) did not result in any significant change in male AGD at e21.5 (Fig. 5.2).

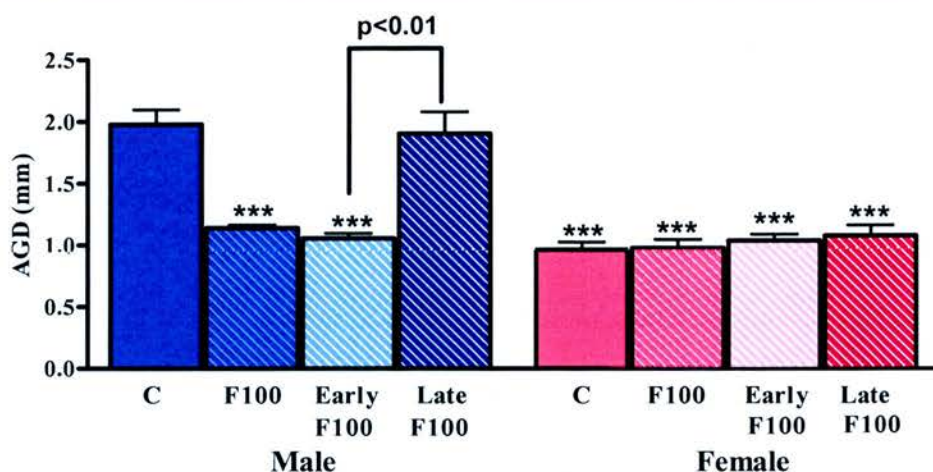


Figure 5.2 Anogenital distance (AGD) in e21.5 male (blue bars) and female (pink bars) fetuses from control (solid bars) and high dose maternal flutamide (100 mgkg^{-1} ; striped bars) litters. Note that AGD was significantly smaller in control female fetuses (solid pink bar) than in males (solid blue bar): AGD in male fetuses exposed to flutamide between e15.5-21.5 (blue striped bar) or between e15.5-17.5 (pale blue striped bar) was reduced compared to control males and was comparable to female AGD. Exposure to flutamide late in WD development (e19.5-21.5, dark blue striped bars) did not affect male AGD. Maternal exposure to flutamide did not alter female AGD (pink bars, $p > 0.05$). *** $p < 0.001$ compared to control male AGD. Values are means \pm S.E.M for 6-23 animals.

Maternal exposure to flutamide (e15.5-21.5 or e15.5-17.5) prevented normal prostate and penis formation in males and resulted in the presence of a vaginal pouch (data not shown) whereas exposure to flutamide late in reproductive development (e19.5-21.5) did not.

Testis descent was unaffected at e21.5 by maternal flutamide exposure (data not shown). Consistent with previous reports (Mylchreest *et al*, 1999; McIntyre *et al*, 2001; Foster and Harris, 2005), these abnormalities were still evident postnatally (data not shown).

5.3.2 Effects on WD development of impaired androgen action during critical windows of development

Exposure to flutamide (100 mgkg^{-1}) between e15.5-21.5 reduced WD development and coiling compared to age matched controls, as detailed earlier (section 3.4.4.1.1). Exposure to flutamide only between e15.5-17.5 (early flutamide) inhibited coiling by e21.5 to a similar extent as did exposure from e15.5-e21.5, whereas coiling was still evident at e21.5 in WDs exposed to flutamide between e19.5-21.5 (late flutamide) with little change compared to age matched controls (Fig. 5.3). This difference was confirmed quantitatively (Fig. 5.4) highlighting that there was no significant difference in luminal length between WDs from

litters exposed to flutamide between e15.5-21.5 and those exposed to flutamide only between e15.5-17.5. Exposure to flutamide late in WD development (e19.5-21.5) did not significantly reduce WD luminal length compared to age matched controls: this was significantly different from WDs exposed to flutamide early in development ($p < 0.001$).

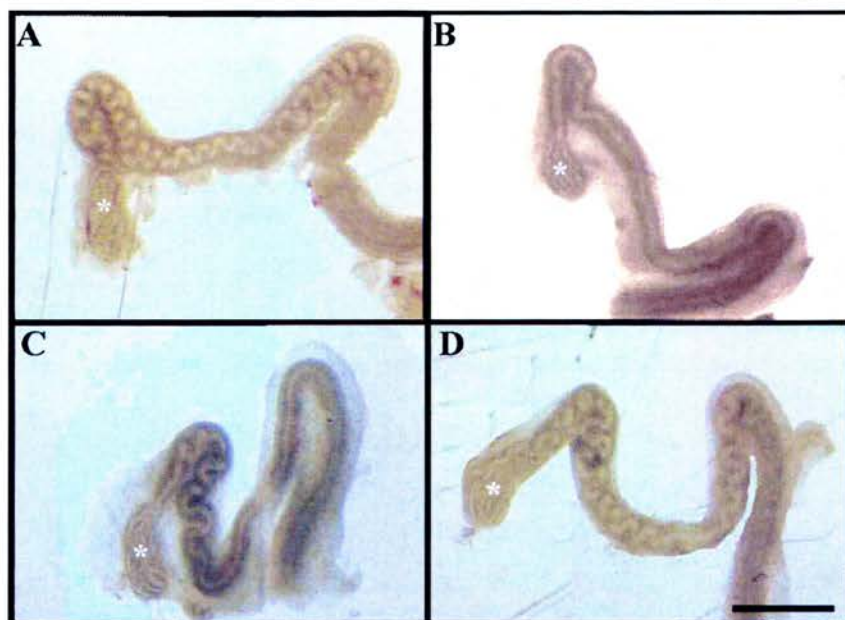


Figure 5.3 Representative Wolffian ducts (WD) at e21.5 from control and flutamide-exposed (100 mgkg^{-1}) fetuses. Note that exposure to flutamide between e15.5-21.5 (B) reduced WD coiling compared to controls (A). Exposure to flutamide between e15.5-17.5 (Early F100, C) reduced WD coiling to a similar extent to exposure to flutamide from e15.5-21.5 (B) while exposure to flutamide only between e19.5-21.5 (D) did not obviously reduced WD coiling compared to age matched controls. * - efferent ducts Scale bar = 1mm

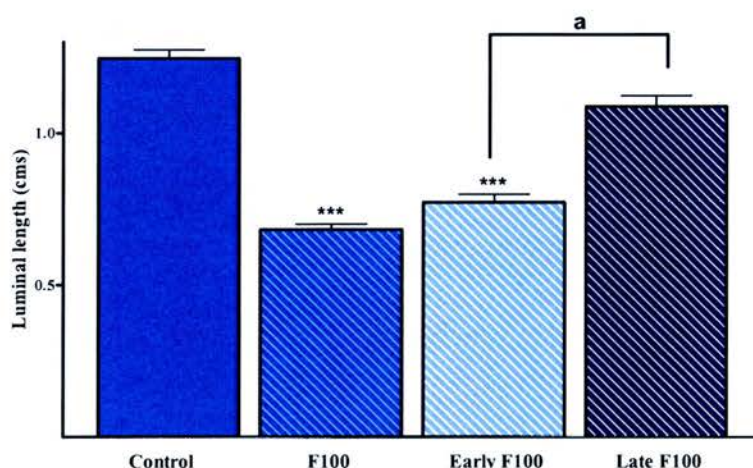


Figure 5.4 Quantification of coiling (= luminal length) of e21.5 WDs from control and flutamide-exposed litters. Note the significant reduction in WD luminal length in animals exposed in utero to 100 mgkg^{-1} (blue striped bar) flutamide from e15.5-e21.5, when

compared to controls (blue bar). Exposure to flutamide from e15.5-17.5 (Early F100; pale blue striped bar) significantly reduced WD luminal length compared to controls. Note that exposure to flutamide between e19.5-21.5 (late F100; dark blue striped bar) did not reduce WD luminal length, compared to age matched controls. Values are mean \pm S.E.M for 8-66 animals per group, *** p <0.001, in comparison to the respective control value, a p <0.001, in comparison to exposure to flutamide from e15.5-21.5.

5.3.3 Androgen receptor expression

Exposure to flutamide either early (e15.5-17.5) or late in WD development (e19.5-21.5) did not prevent expression of androgen receptor (AR) in either the stroma or epithelium at e21.5 (Fig. 5.5, panels B and C, respectively). Further analysis of AR expression using Taqman QRT-PCR confirmed that there was no significant difference either in AR mRNA expression in control compared to flutamide-exposed WDs at e21.5 (Fig. 5.6). Variation was seen in the levels of AR mRNA within each age/treatment however, this was not significant and presumably represents variation between individual WDs.

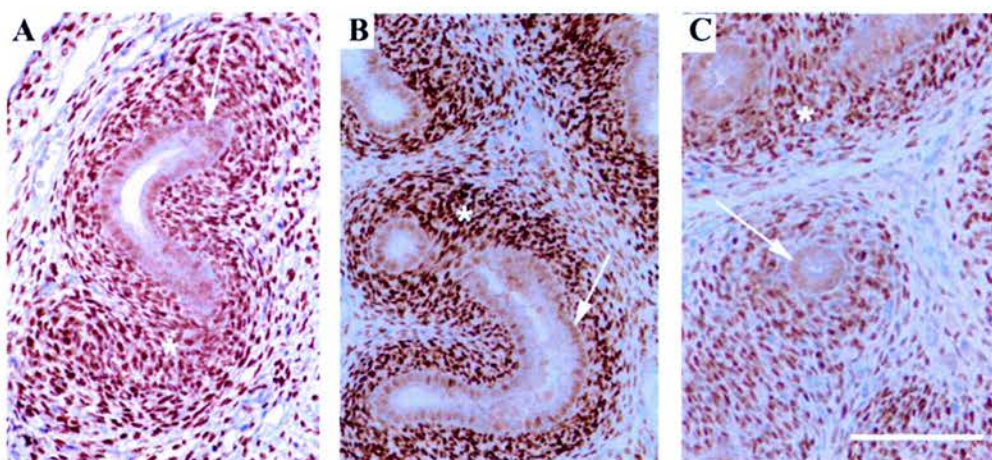


Figure 5.5 Expression of androgen receptor (AR) protein in the Wolffian duct (WD) from control (A) and flutamide-exposed (B – 100 mgkg⁻¹ e15.5-17.5, C – 100 mgkg⁻¹ e19.5-21.5) rats at e21.5. Note that exposure to flutamide, either early (B) or late (C) in WD development, does not alter AR expression in the stroma (*) or epithelium (arrow), compared to age matched controls (A). Scale bar = 100 μ m.

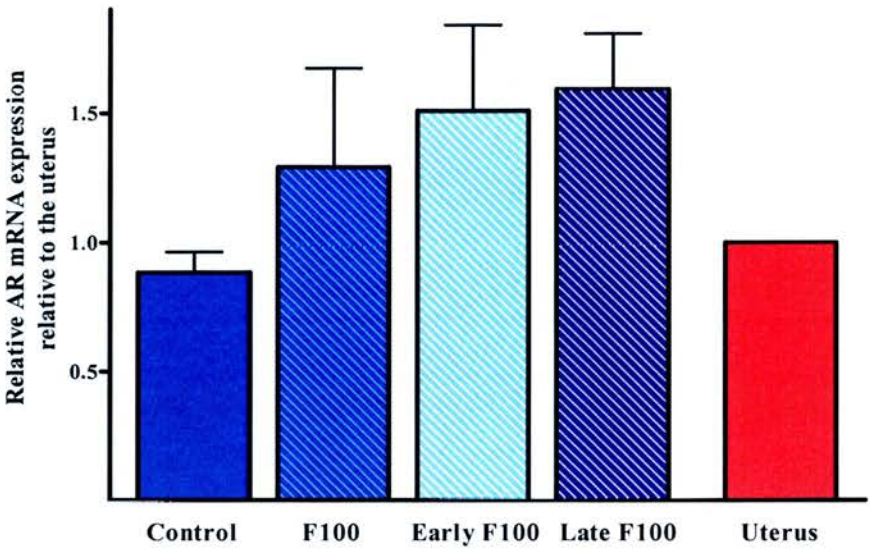


Figure 5.6 Quantitative analysis of AR mRNA in the Wolffian duct at e21.5. No significant difference was seen in AR mRNA expression in WDs from control (solid blue bars) and flutamide-exposed (striped bars – 100 mgkg⁻¹) rats, as determined by Taqman QRT-PCR (means \pm S.E.M, n=3-5 WDs per age/treatment, each from different litters). mRNA expression was corrected for loading and expressed relative to levels in the uterus.

5.3.4 Gross changes to the epithelial compartment

Examination of WDs revealed no obvious histological differences in the epithelium of e21.5 WDs from control fetuses and fetuses exposed to 100 mgkg⁻¹ flutamide late in WD development (e19.5-21.5). However, as highlighted in Fig. 5.7, epithelial abnormalities became apparent by e21.5 in WDs from fetuses exposed to 100 mgkg⁻¹ flutamide early in WD development (e15.5-17.5) including loss of epithelium lining the lumen or flattening of the epithelium compared to that of controls (Fig 5.7). The extent of this flattening varied between WDs but was confirmed quantitatively at e21.5 (Fig. 5.8). These epithelial abnormalities were similar to those observed in WDs from e21.5 fetuses exposed to 100 mgkg⁻¹ flutamide between e15.5-21.5 (section 3.4.5.1).

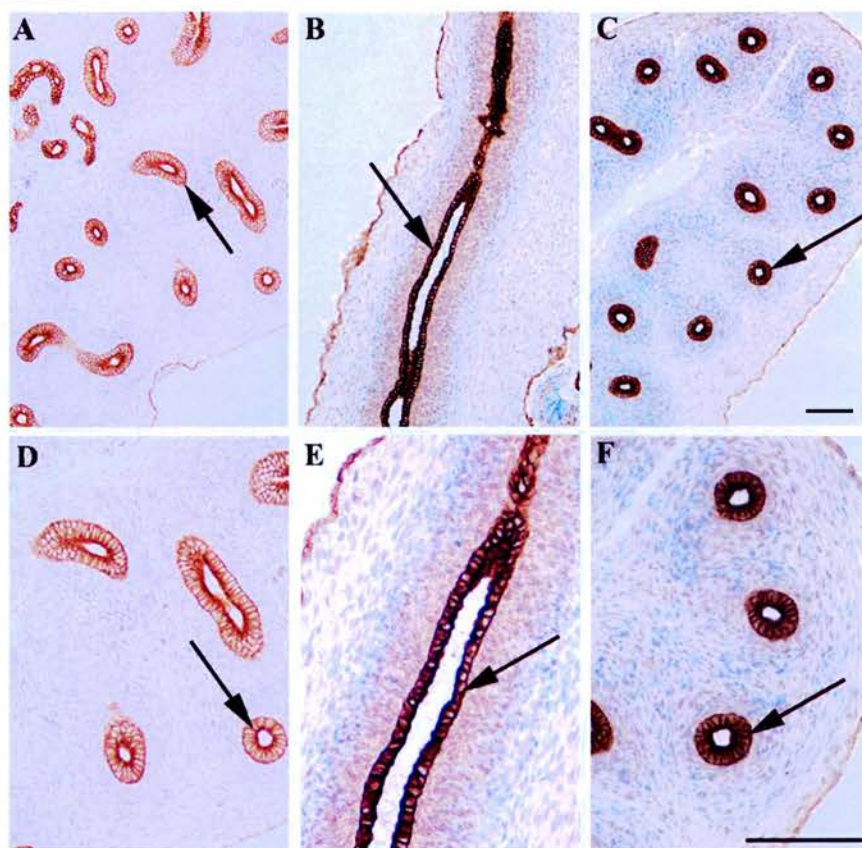


Figure 5.7 Representative gross abnormalities seen in the epithelium of e21.5 WDs (immunostained brown for cytokeratin) from control and flutamide-exposed (100 mg kg^{-1}) litters. Note the apparent reduction in epithelial cell height (arrow) in WDs exposed to flutamide early in development (e15.5-17.5, B & E) but not in WDs exposed to flutamide later in WD development (e19.5-21.5, C & F), compared to age matched controls (A & D). Scale bars = $100 \mu\text{m}$

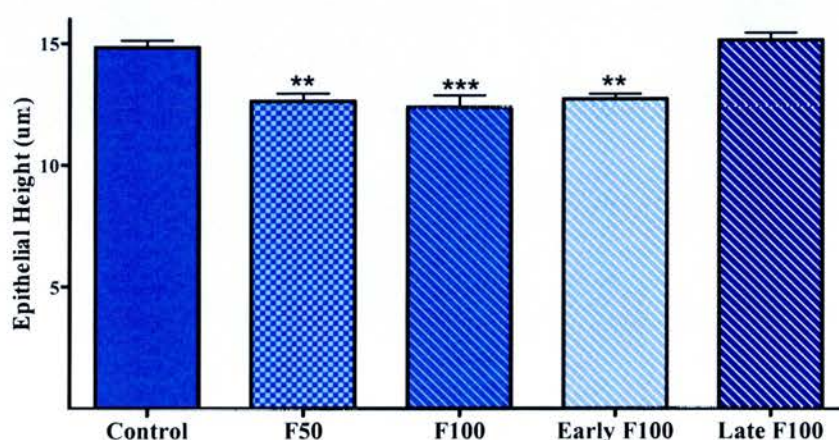


Figure 5.8 Analysis of epithelial cell height in Wolffian ducts (WDs) at e21.5 from control and flutamide-exposed animals. Note that epithelial cell height is significantly reduced in males exposed to flutamide between e15.5-21.5 (blue chequered bar - 50 mg kg^{-1} ; blue striped bar - 100 mg kg^{-1}) and between e15.5-17.5 (Early F100, pale blue striped bar -

100 mgkg⁻¹) but not in males exposed to flutamide late in WD development (Late F100, dark blue striped bar - 100 mgkg⁻¹), compared to age matched controls (blue bar). Values are means ± S.E.M, for 3-19 WDs per age/treatment, each from different litters. ** p<0.01, *** p<0.001 compared to e21.5 controls.

5.3.5 Flutamide-induced loss of Wolffian duct segments

As well as the reduction in coiling, exposure to flutamide from e15.5-21.5 resulted in some WDs appearing incomplete at e21.5 with thinning of the epithelium and missing corpus/cauda segments (detailed in section 3.4.4.1). This phenotype of missing segments was not noted at e21.5 in fetuses exposed to flutamide during short windows either early (e15.5-17.5) or late (e19.5-21.5) in WD development (Table 5.3). However, by pnd42 40% of males exposed to flutamide early in development had incomplete reproductive tracts with missing or incomplete epididymides and/or vas deferentia. By adulthood (pnd70), 50% of males exposed to flutamide early in WD development had incomplete epididymides and/or vasa deferentia (Fig. 5.9). A further 25% of males had underdeveloped or abnormal epididymides/vasa deferentia (Fig. 5.9). This phenotype was similar to that observed in males exposed to flutamide throughout fetal reproductive development in which 83% of males examined had incomplete WD-derived tissues (section 3.4.4.2). In contrast, males exposed to flutamide late in development all had complete epididymides and vasa deferentia at pnd42 and in adulthood (Fig. 5.9 and Table 5.3).

Table 5.3 Percentage of rats with an intact WD (e21.5) or WD-derived tissues (pnd17, pnd42, adult) after exposure to flutamide (100 mgkg⁻¹)

	Control	Flutamide (e15.5-21.5)	Early flutamide (e15.5-17.5)	Late flutamide (e19.5-21.5)
e21.5	100%	89%	100%	100%
Pnd17	100%	87%	100%	100%
Pnd42	100%	50%	60%	100%
Adult	100%	17%	50%	100%

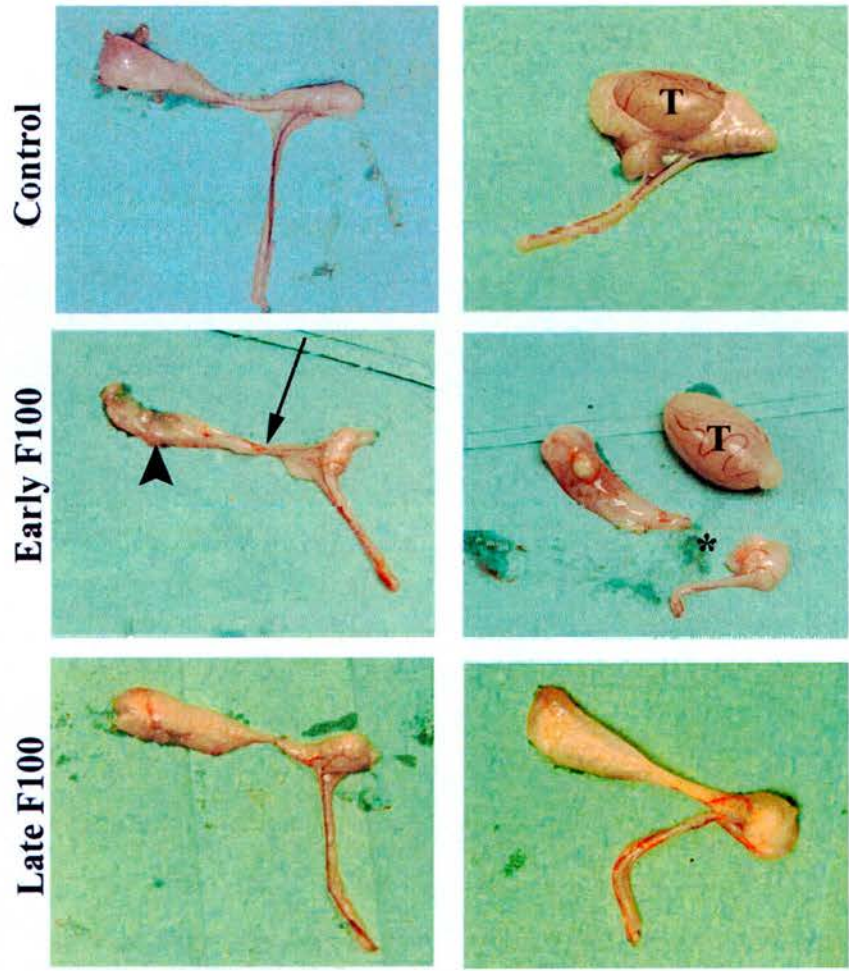


Figure 5.9 Wolffian duct-derived tissues in males from control and flutamide exposed (100 mgkg^{-1}) animals at pnd70. Note the thinning of the corpus (arrow), the vestigial caput segment (arrowhead) and missing corpus (*) in epididymides from males exposed to flutamide early in fetal development (e15.5-17.5), compared to age matched control males (top row). In contrast, the WD-derived structures from animals exposed to flutamide late in development (e19.5-21.5) were complete and appeared normal (bottom row).

5.3.6 Alterations to the basement membrane

In control animals, laminin was immunolocalised to the basement membrane in WDs, forming a ‘ring’ between the epithelium and the stromal compartment (section 4.4.4.1). Exposure to flutamide late in WD development (e19.5-21.5) did not alter laminin expression but flutamide exposure early in WD development (e15.5-17.5) resulted in interruptions to the laminin ‘ring’ and it appeared less defined (Fig. 5.10).

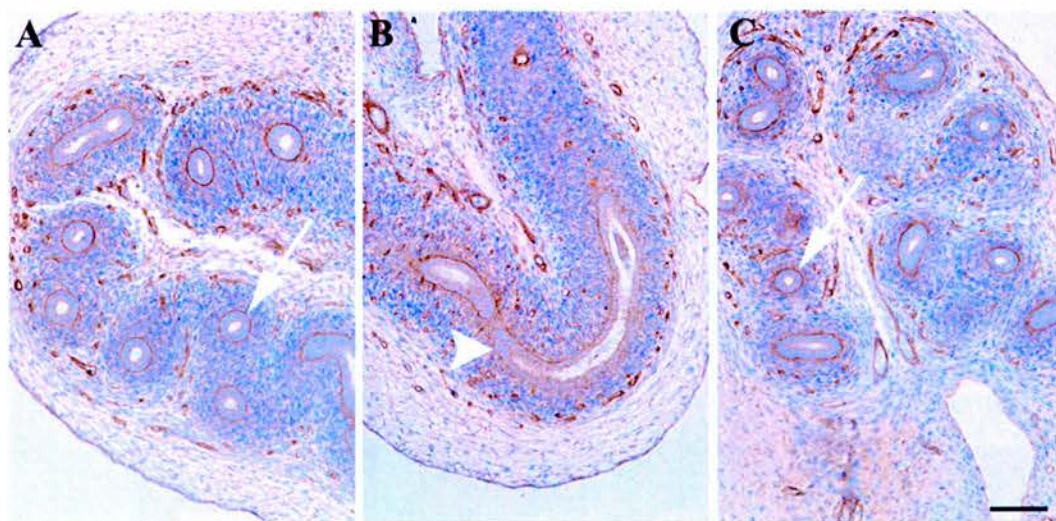


Figure 5.10 *Immunoexpression of laminin in the basement membrane of control (A) and flutamide-exposed (B & C) WDs at e21.5. Note the defined 'ring' of laminin around the base of the epithelium in the control WD (arrow, A). This 'ring' becomes less defined and interrupted in WDs exposed to flutamide early in WD development (e15.5-17.5, arrowhead, B) but not in WDs exposed to flutamide late in WD development (e19.5-21.5, arrow, C). Scale bar = 100 μ m*

5.3.7 Stromal cell differentiation

Immunostaining for smooth muscle actin (SMA), a smooth muscle cell marker specifically expressed in the inner stromal compartment, suggested a reduction in the SMA-positive layer in WDs from e21.5 fetuses that had been exposed to flutamide early in development, compared to age matched controls (Fig. 5.11). No obvious change was noted in this SMA positive layer in WDs from males exposed to flutamide during the late window (Fig. 5.11). The SMA positive stromal compartment varied considerably in breadth between sections of WDs so SMA protein expression in WDs was quantified by Western blot analysis (Fig. 5.12). This confirmed that SMA protein levels in WDs from males exposed to flutamide early in development, but not those from late window flutamide fetuses, were significantly different from that in age-matched controls (Fig. 5.12). This is consistent with the reduction in SMA protein levels observed in WDs from fetuses exposed to flutamide (100 mgkg^{-1}) between e15.5-21.5 (detailed in section 4.4.2.2.2).

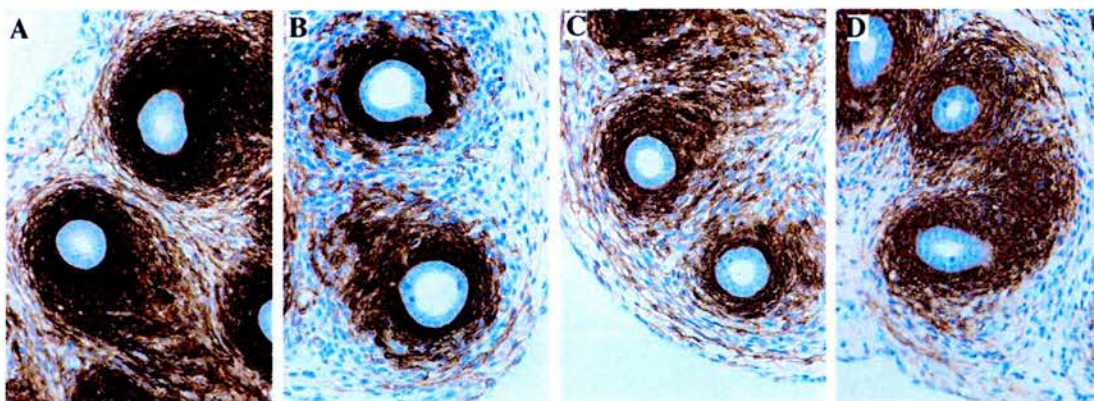


Figure 5.11 Comparative expression of smooth muscle actin (SMA, *) at e21.5 in Wolffian ducts (WDs) from controls (A) and animals exposed in utero to 100 mgkg⁻¹ flutamide between e15.5-21.5 (B), e15.5-17.5 (C) or e19.5-21.5 (D). Note the apparent reduction in the depth of the SMA positive layer in WDs from animals exposed to flutamide either between e15.5-21.5 (A) or e15.5-17.5 (C), but not in WDs from males exposed to flutamide between e19.5-21.5 (D).

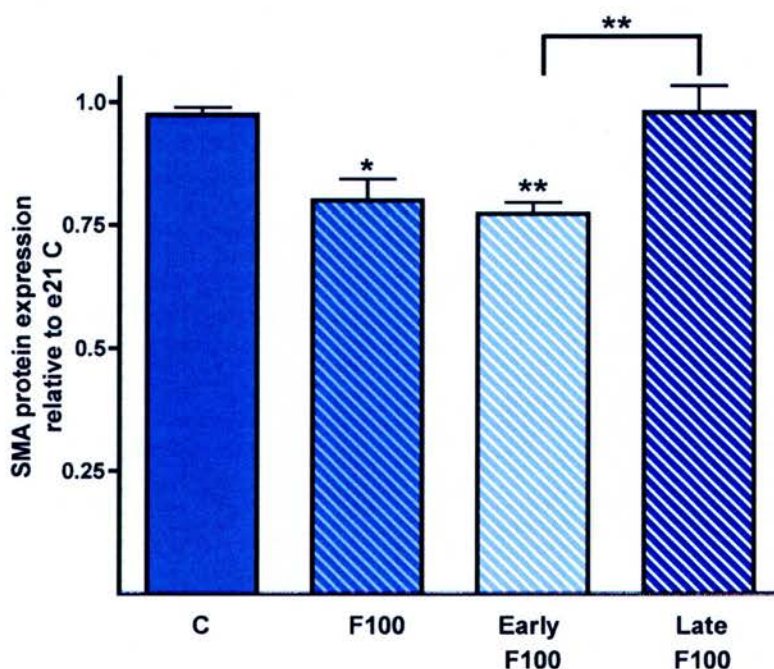


Figure 5.12 Quantitative data comparing expression of smooth muscle actin (SMA) protein at e21.5 in WDs from control (C) and flutamide-exposed animals. Note the reduction in SMA protein expression in WDs from fetuses exposed to flutamide between e15.5-21.5 (striped bar) or e15.5-17.5 (pale blue striped bar) while no obvious difference can be seen in SMA protein expression in WDs from fetuses exposed to flutamide between e19.5-21.5 (dark blue striped bar), compared to age matched controls (solid blue bar). n = a pool of 3-5 WDs per age/treatment, from at least 3 different litters. Loading was corrected using β -tubulin protein expression.

5.3.8 Vimentin expression

Vimentin protein was immunolocalised to the WD stroma of both control and flutamide-exposed fetuses at e21.5 (Fig. 5.13). Vimentin was also detected at the basolateral edges of epithelial cells in the distal corpus and cauda of the future epididymis in control WDs at e21.5. Exposure to flutamide did not affect stromal vimentin expression but altered the distribution of vimentin in the epithelium. In WDs from fetuses exposed to flutamide (100 mg kg^{-1}) between e15.5-21.5 or between e15.5-17.5, vimentin 'spiking' was apparent in the caput of many WDs at e19.5 and e21.5, as well as in the cauda (Fig. 5.13). This was rarely seen in the caput of WDs exposed to flutamide late in WD development (e19.5-21.5).

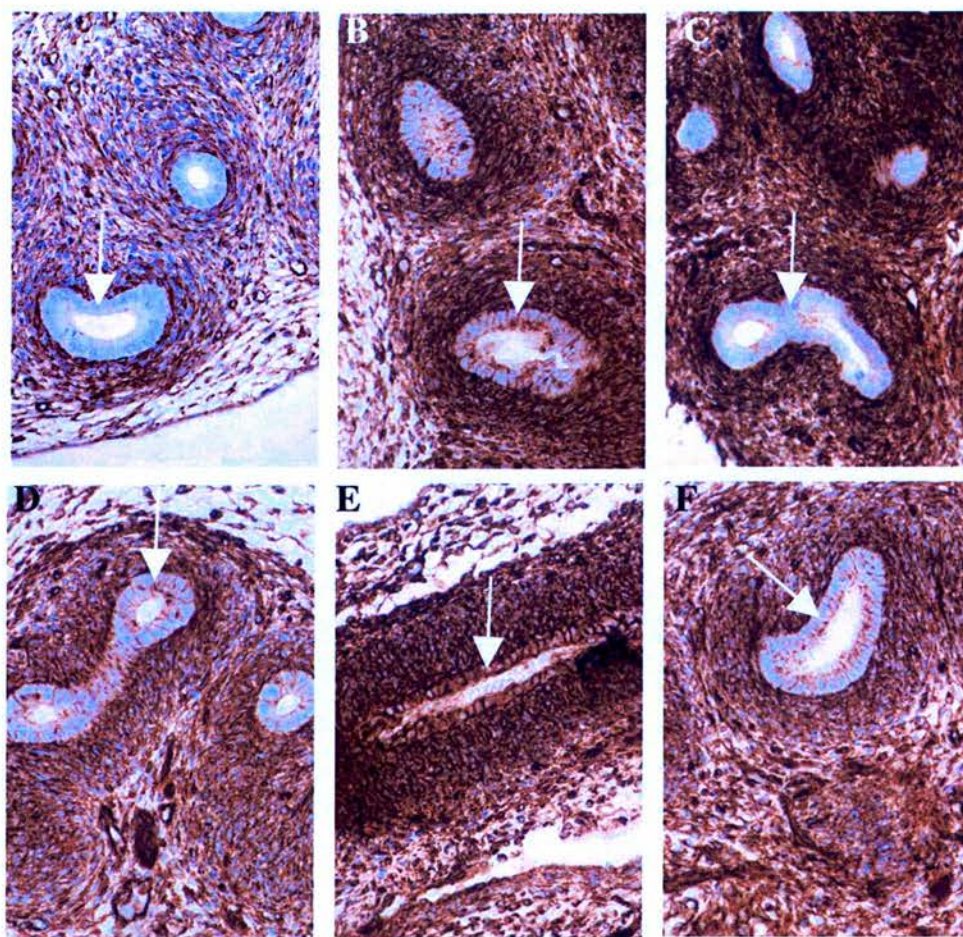


Figure 5.13 Immunolocalisation of vimentin protein in e21.5 Wolffian ducts from control and flutamide-exposed animals. Note that vimentin expression in the stroma did not vary with flutamide treatment (brown). Vimentin was also detected in the epithelium (arrow) in the WD cauda (D), but not the caput (A), in control animals. This vimentin 'spiking' in the epithelium became apparent in the caput (B) as well as in the cauda (E) in fetuses exposed to flutamide between e15.5-17.5. This was not seen in the caput of WDs exposed to flutamide late in development (C) but was still detected in the cauda (F).

5.4 Discussion

In males, androgen action is believed to stabilise the Wolffian duct (WD) during fetal life, allowing it to differentiate subsequently into its adult derivatives, the epididymis, vas deferens and seminal vesicles (George, 1994; Huhtaniemi, 1994). In the female rat, the lack of androgens results in the WD naturally regressing between e16.5 and e18.5 (chapter 6). This suggests that WD stabilisation occurs between e15.5, the time of testosterone production onset, and e18.5 while studies in our rat colony have shown that the window for WD morphological differentiation is between e19.5 and birth, coinciding with the peak in testosterone seen in male rats at e19.5 (chapter 3)(Warren *et al*, 1972).

Flutamide was used to interfere with androgen action as it has been shown to be a strong competitive AR antagonist and exposure during fetal development interrupts male reproductive development (Imperato-McGinley *et al*, 1992; Bentvelsen *et al*, 1995; Mylchreest *et al*, 1999; McIntyre *et al*, 2001; Hotchkiss *et al*, 2002). It is worth noting that flutamide is quickly cleared from the body and has a plasma half-life of 5-6 h in man (Neri and Monahan, 1972) thus suggesting that fetuses are not exposed to flutamide for more than 24 h after the final gavage. Studies reported in chapter 3 have confirmed the role for androgens in male fetal development since male fetuses from dams exposed to 100 mgkg⁻¹ flutamide between e15.5-21.5 had no prostate, reduced AGD and impaired WD development. These abnormalities persisted into adulthood confirming that the patterning and establishment of the reproductive tract is set up in the fetus, and interfering with androgen action during this period permanently alters reproductive tissues. It was assumed that androgens are essential during the period of both WD stabilisation and morphological differentiation: in order to test this hypothesis, fetuses were exposed to flutamide either during the window of WD stabilisation (e15.5-17.5) or during WD morphological differentiation (e19.5-21.5). Since exposure to 100 mgkg⁻¹ flutamide between e15.5-21.5 did not prevent stabilisation of the male WD but impaired WD coiling, it was expected that impaired androgen action late in development would allow 'rescue' of the WD but would prevent initiation of WD coiling whereas exposure to 100 mgkg⁻¹ flutamide early in development would not have any major impact on the developing WD. Contrary to these hypotheses, reduced androgen action early in male reproductive tract development (e15.5-17.5) resulted in a similar phenotype to exposure to flutamide during the entire window of male fetal reproductive development (e15.5-21.5) with males having a vaginal pouch instead of a normal prostate, impaired WD development and with their AGD reduced to the length found in normal females. This phenotype was not observed in male fetuses exposed to

maternal flutamide later in development (e19.5-21.5). These results suggest that prostate formation, WD patterning and masculinisation of AGD is established between e15.5 and e17.5, several days before any sign of WD morphological differentiation, and that high levels of androgen action later in fetal development are not essential for maintaining this male phenotype. This is in agreement with previous studies exposing pregnant rats to a single dose of flutamide (50 mg kg^{-1}) on e16, 17, 18 or 19 which showed that exposure to flutamide on e16 or 17 resulted in missing epididymides in adults whereas exposure later in development (e18 or 19) only resulted in smaller epididymides (Foster and Harris, 2005). Furthermore, they highlighted that the peak incidence of abnormal prostate development was noted in adults exposed to flutamide on e17 or 18, similar to our observations in males exposed to flutamide between e15.5-17.5. Foster and Harris reported that exposure to a single dose of flutamide resulted in a similar range of reproductive abnormalities as exposure to flutamide throughout the period of reproductive development (e12-21) however, the incidence was lower (Foster and Harris, 2005). This suggests that a single exposure to flutamide can interfere with normal reproductive development but at a lower incidence and even showing a less severe phenotype. This reduced incidence was not noted in our studies since exposure to flutamide for 3 days early in development (e15.5-17.5) resulted in a similar number of adult males with reproductive tract abnormalities as exposure throughout reproductive development. It was surprising that high levels of androgen action did not appear to be essential during the window of morphological differentiation of the WD (e19.5-21.5) but rather once the pattern of WD development was established by early androgen exposure, the WD continued to development along the normal pathway, even if androgen action was reduced by exposure to flutamide. It is worth noting that, as discussed in chapter 3, exposure to this level of flutamide does not completely block androgen action, therefore we cannot completely rule out a role for low levels of androgens during this late window of WD development (e19.5-21.5). Studies carried out in the mouse have shown that exposure to flutamide on e19 and 20 did not result in any obvious reproductive abnormalities or infertility in adulthood whereas exposure on e11-15 or e15-20 resulted in smaller epididymides and infertility and lack of a prostate, respectively (Silversides *et al*, 1995). These studies again confirm the long term deleterious effects of short-term androgen blockade. It is not clear if androgens play any role late in fetal male reproductive development and further investigation of more endpoints would be required in males from litters exposed to flutamide between e19.5-21.5.

Studies previously published (Silversides *et al*, 1995; Foster and Harris, 2005) and those reported in this thesis, have shown that interfering with androgen action during defined

windows of fetal development have varied effects on the male reproductive tract. Exposure to flutamide (100 mg kg^{-1}) during the entire window of male reproductive development resulted in incomplete WDs in 11% of e21.5 males: the prevalence of this abnormality increased around puberty to 50% of males and increased again in adults to 83%. This is in agreement with previous studies showing that exposure to anti-androgenic compounds *in utero* results in a high frequency of epididymal malformations in adult rats (Imperato-McGinley *et al*, 1992; Mylchreest *et al*, 1999; McIntyre *et al*, 2001; Turner *et al*, 2003; Foster and Harris, 2005). The reasons for this increased prevalence as development proceeds were unclear however it is hypothesised to be due to failure to establish normal patterning of the WD in the fetus. Exposure to flutamide either early or late in WD development did not result in incomplete WDs during fetal life in any fetuses examined, but this may be due to the relatively low number of litters studied. Postnatal males exposed to flutamide late in development (e19.5-21.5) did not show any signs of this phenotype with all reproductive tracts studied appearing morphologically normal at all ages studied (pnd17, 42 and 70). On the other hand, 40% of pubertal males (pnd42) examined that had been exposed to flutamide early in WD development (e15.5-17.5), had incomplete epididymides and/or vas deferentia. This increased to 50% by adulthood. The slightly lower incidence of incomplete WD-derived structures seen in animals exposed to flutamide early in development (e15.5-17.5) compared to the incidence noted in males exposed to flutamide throughout fetal reproductive development (e15.5-21.5), may be due to the lower number of litters examined in the early exposure group: an increased percentage of incomplete reproductive tracts may be seen in the early exposure group if more animals were examined. This data again confirms the role for androgens early in WD development (e15.5-17.5) to establish the pattern of WD development and highlights that interfering with this patterning during fetal life has long term consequences for the adult male reproductive tract.

In order to confirm that the reduced WD development seen in animals exposed to flutamide early in fetal development was due to similar mechanisms as that seen in animals exposed to flutamide throughout reproductive development (see chapter 3 and 4), various endpoints were examined. As discussed in chapter 4, exposure to flutamide did not alter expression of the androgen receptor (AR) in the stroma or epithelium of the WD during fetal life. Similarly, exposure to flutamide during either defined window (early or late) did not interrupt AR expression, confirming that these WDs are still capable of responding to androgens. This is in contrast to findings by Bentvelsen and colleagues who were unable to detect AR protein by immunohistochemistry in WDs from e21.5 fetuses exposed to 100

mgkg⁻¹ flutamide (Bentvelsen *et al*, 1995). This difference may be due to differences in rat strain or to the use of different anti-AR antibodies and immunohistochemistry techniques.

Furthermore, exposure to flutamide between e15.5-21.5 resulted in reduced epithelial cell height and abnormal epithelial development in the WD by e21.5 (see chapters 3 and 4). This was not found in WDs exposed to flutamide late in WD development (e19.5-21.5) but was seen in WDs exposed to flutamide early in development. These effects on the epithelium were not evident until e21.5, thus suggesting that the epithelium initially forms normally in flutamide-exposed animals but degenerates during differentiation, possibly due to impaired androgen-driven signalling between the stroma and epithelium. Since no effect on epithelial cell height or loss of epithelium was found after exposure to flutamide between e19.5-21.5, only in animals exposed to flutamide between e15.5-17.5, this again confirms the early role for androgens in establishing the fate of the epithelial cells in WD development.

Another deleterious effect caused by flutamide exposure between e15.5-21.5 was interruption of the basement membrane. Laminin forms a defined 'ring' around the base of the epithelium and plays an essential role in anchoring epithelial cell surfaces to the basement membrane (see chapter 4). Exposure to flutamide early, but not late, in WD development also interrupted the basement membrane with the laminin 'ring' appearing incomplete and less defined, as seen in WDs from animals exposed to flutamide between e15.5-21.5. Interruption to the basement membrane might explain the abnormal epithelium in sections of the WD and the appearance of flattened mesenchymal-like cells (as discussed in chapter 4).

The early mesenchymal cell marker, vimentin, was constitutively expressed in the WD stroma and was not altered by exposure to flutamide either throughout fetal reproductive development (e15.5-21.5, see chapter 4) or flutamide-exposure only during defined windows. In control animals, vimentin was also expressed on the basolateral boundary of the epithelial cells along the full length of the WD at e15.5 and becomes later restricted mainly to the cauda by e21.5: this restriction of vimentin expression in the epithelium may reflect vimentin being replaced by another intermediate filament in a cranio-caudal direction as development and differentiation proceeds. Exposure to flutamide between e15.5-21.5 disrupts this cranio-caudal loss of epithelial vimentin reflecting the failure of the epithelium to differentiate fully, as discussed in chapter 4. This pattern was again seen in WDs exposed to flutamide between e15.5-17.5 with vimentin still strongly expressed in the WD epithelium in the caput as well as in the cauda, in contrast to controls. Conversely, vimentin was rarely detected in the caput epithelium in WDs exposed to flutamide late in WD development. These data provide further evidence for a key role for androgens between e15.5-17.5, rather

than during WD morphological differentiation (e19.5-21.5), in mediating epithelial differentiation and WD development. This might explain the reduced epithelial cell height and loss of attachment to the basement membrane seen in WDs exposed to flutamide early, but not late, in WD development.

Finally, as discussed in chapter 4, smooth muscle stromal differentiation occurred during fetal life with SMA detected in the stroma immediately proximal to the epithelium in the WD corpus by e18.5: this was impaired by exposure to flutamide between e15.5-21.5. A similar reduction was seen in the level of SMA protein expression in WDs recovered from fetuses exposed to flutamide early in WD development (e15.5-17.5), but not in WDs from fetuses exposed to flutamide in the late window (e19.5-21.5). This suggests that WD development and smooth muscle differentiation are mediated by androgen action between e15.5-17.5, even though the effects are not seen until several days later.

5.5 Conclusions

These studies highlighted that the pattern of WD coiling and its subsequent ability to develop fully during postnatal life is established early in fetal life (e15.5-17.5) as reduced androgen action during this time-window inhibited coiling at e21.5 to the same extent as did exposure from e15.5-e21.5 and resulted in a similar high incidence of epididymal loss/abnormalities in late puberty and adulthood. Exposure later in fetal life (e19.5-21.5), the period in which WD morphological differentiation occurs, did not impact on WD coiling at e21.5 or subsequent epididymal development postnatally: this suggests that WD patterning is already established by e19.5 and is no longer dependent on high levels of androgen action. The critical window for high levels of androgen action in establishing the pattern of WD development is therefore between e15.5-e17.5.

6 Investigation of Wolffian duct stabilisation and its dependence on androgens

6.1 Introduction

The studies presented so far in this thesis highlight that the pattern of WD coiling and its subsequent ability to develop fully during postnatal life is dependent on androgen action and is established early in fetal life (e15.5-17.5): patterning of WD differentiation has already been established prior to any signs of morphological differentiation of the WD (i.e. e19.5-20.5), and by this age, the WD is no longer dependent on high levels of androgen action. Studies in chapter 3 suggested that patterning of WD differentiation is more susceptible to reduced androgen action than is stabilisation. One interpretation of these studies is that the dose of flutamide administered does not completely block androgen action and so does not interfere with WD stabilisation and cause it to regress as occurs in the female. This suggests that WD stabilisation is dependent on lower levels of androgen action than that required for differentiation. Alternatively, WD stabilisation and differentiation could be regulated by different mechanisms, thus highlighting WD development as a bi-phasic process. Insight from models in which androgen action is more completely blocked may further our understanding of WD development.

Since the WD initially forms in the female then subsequently regresses due to the lack of androgens, females offer a natural model in which to study the impact of a complete lack of androgen signalling on the fetal WD (George, 1994). The female WD regresses in a cranio-caudal direction and has almost completely regressed by e19 in the rat (Jirsova and Vernerova, 1993). The mechanisms involved in the regression of the female WD are not fully understood but similarities can be seen between the cellular processes suggested to be involved and those reported in this thesis to be induced in the male WD by flutamide exposure (chapter 4). Previous research has shown that the WD diameter decreases and the epithelial cells lose their polarity and degenerate: it has been suggested that these epithelial cells are lost due to apoptosis as well as a possible role for epithelial-mesenchymal transformation (Inomata *et al*, 1989; Jirsova and Vernerova, 1993). This is similar to the process observed in the regressing MD, in which the basement membrane is disrupted, epithelial cell size decreases, the mesenchyme condenses and the epithelium subsequently degenerates (Dyche, 1979; Ikawa *et al*, 1984; Inomata *et al*, 1989; Austin, 1995). This is thought to be driven by paracrine mechanisms originating in the mesenchyme as a result of a loss of ECM and results in apoptosis and possibly epithelial-mesenchymal transformation

(Dyche, 1979; Inomata *et al*, 1989; Austin, 1995; Allard *et al*, 2000; Xavier and Allard, 2003).

It is presumed that the female WD is identical to the male WD and would develop in the same way if exposed to androgens, however this is a presumption and has not been proven. Testicular feminised (Tfm) mice provide a model in which to examine the effects of a complete lack of androgens on male WD development since these mice have an X-linked recessive mutation which causes a complete insensitivity to androgens due to a defect in AR protein, not androgen production (Attardi and Ono, 1974; Gehring and Tomkins, 1974) (section 1.5.2.1). Similar AR mutations can be identified in humans resulting in end-organ androgen resistance termed androgen insensitivity syndrome (AIS) (Quigley *et al*, 1995; Ahmed *et al*, 2000). Both of these models result in genetic males with normal testes and testosterone production but with phenotypic female genitalia, though the clinical observations in AIS patients can vary considerably depending on the degree and location of the AR mutation (Lyon and Hawkes, 1970; Hughes and Patterson, 1994; Patterson *et al*, 1994; Hannema *et al*, 2004). Complete AIS (CAIS) patients and Tfm mice have no obvious WD-derived structures in adulthood suggesting that the fetal WD is not rescued but, to the best of my knowledge, current literature has not examined the status of the fetal WD in either model. Furthermore, it has been noted by several researchers that rudimentary internal genitalia can persist in some AIS individuals (Morris, 1953; Bale *et al*, 1992; Boehmer *et al*, 2001). This is believed to be due to the failure of the WD to completely regress. However, Hannema and colleagues (2004) have shown the persistence of well developed epididymides and vasa deferentia in some CAIS patients. These patients all had mutations in the AR ligand binding domain and showed residual transcriptional activity *in vitro*, unlike ARs with mutations in the DNA binding domain which completely lacked transcriptional activity (Hannema *et al*, 2004). Together, these data suggest that WD development is more complex than previously presumed and may not simply depend on the presence of androgens and a functional AR: this therefore warrants further investigation to examine the mechanisms and regulatory factors in WD stabilisation and differentiation.

These models provide systems in which to examine the complete loss of androgen action in the male and female WD but exposure to mixtures of anti-androgenic compounds can provide a tool with which to reduce further androgen action and so examine the dose-dependent effects on male WD development (discussed in section 1.5.2.2). Maternal exposure to anti-androgenic compounds during gestation results in varying degrees of impaired masculinisation of the male offspring, some of which can be observed in the fetus and others are not evident until adulthood. For example, maternal exposure to Di(n-butyl)

phthalate (DBP) reduces testicular testosterone production in the male fetus thus interfering with normal male reproductive tract development (Mylchreest *et al*, 1998; Mylchreest and Foster, 2000; Mylchreest *et al*, 2000; Parks *et al*, 2000; Foster *et al*, 2001; Fisher *et al*, 2003). Conversely, flutamide exposure has no effect on androgen production but prevents transcriptional activation of AR in target tissues (Peets *et al*, 1974). Therefore, maternal exposure to flutamide dose-dependently prevents normal masculinisation of male fetuses resulting in varying degrees of WD differentiation, maldescent of the testis, feminisation of external genitalia and missing prostates in adulthood (Imperato-McGinley *et al*, 1992; McIntyre *et al*, 2001). Combined exposure to both these compounds would theoretically result in a more complete reduction in androgen action by interfering with both androgen production and action and therefore would provide a system in which to examine whether WD development depends on a threshold of androgen action and whether WD regression only occurs due to a complete lack of androgen action.

6.1.1 Aims

To investigate the pattern and timing of WD regression in the female rat.

To compare the mechanisms of natural regression of the WD in the female with the mechanisms of flutamide-induced WD abnormalities.

To investigate if functional inactivation of the AR (Tfm) results in complete regression of the male WD, as occurs naturally in females.

To establish an *In utero* model to investigate more complete reduction in androgen action in rats and investigate the subsequent impact on WD development. Does this further reduction in androgen action result in regression of the WD as occurs naturally in females?

6.2 Methods

6.2.1 Natural regression studies

In order to study the exact timing of WD regression in females, matings were set up to allow collection of fetuses at 6 hourly intervals between e16.5 and e18.5 (i.e. e17.0, e17.25, e17.5, etc). Time-matings were set up to allow accurate calculation of their mating within a 1 h time window. One stud male and one female rat were paired together in grid bottom cages at a precise time and monitored hourly for the presence of a vaginal plug. Detection of a copulatory plug was taken as evidence of mating: this was defined as embryonic day 0.5 (e0.5). Once a positive mating was detected, the female was removed from the male rat.

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation and fetuses were recovered at 6 hourly intervals between e16.5-18.5 (n= 15litters). Fetuses were killed by decapitation before reproductive tracts were recovered by microdissection and photographed using a Leica ICA camera (section 2.3.2.1). Reproductive tracts were analysed microscopically at the time of dissection, for any gross morphological abnormalities and tissue was fixed in Bouin's, as detailed in section 2.4.

6.2.2 *In utero* treatments

As detailed in section 2.2, pregnant dams were randomly allocated to treatment groups and dosed once daily by gavage with 1 mlkg⁻¹ maternal bodyweight of the various treatments detailed below. Dams were dosed between e13.5-e21.5, or until the day prior to cull if this was before e21.5. This window of exposure was selected to encompass the onset of androgen action at e15.5 and the period of fetal male reproductive development (e15.5-21.5) according to Warren et al (1972). This dose of flutamide was selected based on results previously reported in chapters 3 and 4 highlighting that fetal exposure to flutamide induced reproductive tract abnormalities in both fetal life and adulthood. The Di-n-butyl phthalate (DBP) dose was selected based on studies in our lab investigating the effects of DBP on the reproductive tract (Fisher *et al*, 2003; Mahood *et al*, 2005).

Control:	1ml corn oil/2.5% DMSO e13.5-21.5, (n=6 litters)
Flutamide:	100mgkg ⁻¹ in 1ml corn oil/2.5% DMSO, e15.5-21.5 (n=6 litters)
DBP:	500 mgkg ⁻¹ in 1 ml corn oil, e13.5-e21.5 (n=9 litters)
Flutamide + DBP:	100mgkg ⁻¹ flutamide in 1ml corn oil/2.5% DMSO, e15.5-21.5
	500 mgkg ⁻¹ DBP in 1 ml corn oil, e13.5-e21.5 (n = 3 litters)

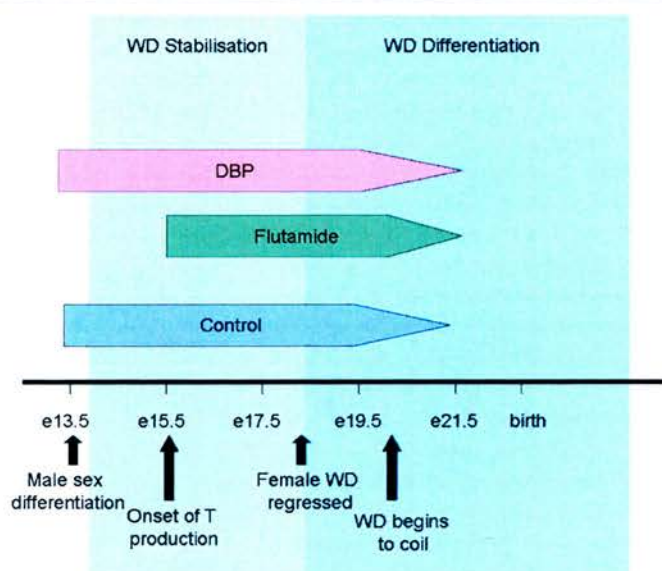


Figure 6.1 Summary of the maternal treatment regimes used.

6.2.3 Tissue collection

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation and fetuses were recovered at e18.5 and e21.5. Fetuses were killed by decapitation before reproductive tracts were recovered by microdissection and photographed using a Leica ICA camera (section 2.3.2.1). Reproductive tracts were analysed microscopically, at the time of dissection, for any gross morphological abnormalities and tissue was either fixed or frozen, as detailed in section 2.4.

6.2.4 Luminal length analysis

Differentiation of e21.5 WDs was quantified by digitally measuring the luminal length of the epididymal segment of WDs from control and treated animals, as described in section 2.8.

6.2.5 Testosterone analysis

Effects of *In utero* treatments on testosterone levels were quantified by measuring the levels of testosterone in whole testis or ovary homogenates at e17.5. Protocols were already established in house for these hormone assays (Atanassova *et al*, 1999).

6.2.5.1 Extraction of testosterone from whole organs

The whole tissue homogenates required an extraction step to free any testosterone bound in the tissue to proteins such as albumin. Whole fetal testes or ovaries were homogenised in 0.5ml ice cold 0.1M PBS buffer and incubated on ice for 1 h: 100µl of the homogenate was

placed in a glass test-tube and vortexed for 5 min with 2 ml diethyl ether. The shaken tubes were placed in a bath of methanol and dry-ice to freeze the aqueous portion, revealing the unfrozen organic portion which was decanted into clean tubes. These tubes were left overnight in a fume hood to enable the organic solvents to evaporate. The dry residue was resuspended in 500 μ l assay buffer and left for 30 min to solubilise: samples were then stored at -20°C until ready to be quantified.

6.2.5.2 Radioimmunoassay (RIA) for testosterone

The radioimmunoassay was performed by Ian Swanson in the HRSU Assay lab, for which I am extremely grateful. This method relies on competition between a known concentration of radiolabelled antigen (testosterone with I^{125} , APB Biotech, UK) against the unlabelled endogenous testosterone present in the test sample. The more testosterone in the test sample, the lower the level of binding by the labelled testosterone. Radiolabelled testosterone was incubated with the test sample extract for 3 h at room temperature. The sample was then incubated overnight at 4°C with a secondary antibody (Donkey-anti-sheep DAGS), raised against the sheep IgG. The antibody binds to the testosterone to form a complex, resulting in the formation of a stable precipitate. 2ml of wash buffer was added and the amount of radiation in the precipitate was measured. The sample values were compared to a standard curve of known antigen concentrations. A gamma counter (Multigamma 1261, LKB Wallac, Turku, Finland) was used to measure residual I^{125} . This assay is precise and sensitive with a low intra-assay coefficient of variance ($<10\%$). In each run, method blanks, quality control (QC) samples (spiked buffer), and standards were analysed along with the samples. Data were expressed as pg testosterone per 100 μ l sample. Results were analysed using a specifically designed computer programme (AssayZap, BioSoft, Cambridge, UK). For presentation purposes, data were converted to ng/ml sample ($= \text{pg}/100\mu\text{l} \times 0.01$).

6.2.6 Immunohistochemistry

Immunohistochemistry was performed on isolated rat reproductive tracts fixed in Bouin's, using standard avidin peroxidase protocols in order to highlight the location of proteins of interest, as detailed in section 2.6.1.

Briefly, sections were cut from paraffin blocks and mounted on glass slides. Sections were then dewaxed and rehydrated then pressure cooked in citrate buffer if required (Table 6.1). Non-specific binding was blocked before adding the appropriate primary antibody overnight, as detailed in Table 6.1. A biotin labelled secondary antibody was used that had been raised against a species-specific sequence on the primary antibody. This biotinylated secondary

antibody signal was amplified by incubation with avidin-biotin conjugated with horseradish peroxidase and the signal was localised using DAB. Sections were counterstained with haematoxylin and mounted with glass coverslips. Further detailed protocols can be found in section 2.6.1.

Table 6.1 Summary of primary antibodies used for immunohistochemistry

Target Antigen	Source	Dilution	Retrieval	Host Species
Androgen Receptor	Santa Cruz	1:50	Citrate	Rabbit
Smooth Muscle Actin	Sigma	1:10000	None	Mouse
Pan-cytokeratin	Sigma	1:200	Citrate	Mouse
Laminin	Abcam	1:100	Citrate	Rabbit
Desmin	DAKO	1:400	Citrate	Mouse

6.2.7 Immunostaining of mitotic cells

WD sections from control and DBP + flutamide-exposed rat fetuses at e18.5 and e21.5 were immunostained for phospho-histone H3 (Upstate Biotechnology, Dundee, UK), a mitotic marker, using a Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK) and a polymer high contrast programme. Briefly, after high pressure antigen retrieval, slides were peroxidase blocked for 5 min, incubated for 2 h with the primary antibody diluted 1:1000 in the diluent supplied and then with the post-primary reagent for 15 min. Control sections were incubated with diluent alone to confirm antibody specificity. Sections were then incubated with the polymer reagent for 15 min to increase sensitivity of detection prior to DAB detection for 10 min, counterstained in haematoxylin for 5 min, dehydrated and mounted.

6.2.8 Analysis of apoptosis

Cleaved caspase 3 immunostaining was performed on WDs from control and treated rat WDs using standard methods, as detailed in section 2.6.1, to stain for apoptotic cells. Very few cleaved caspase 3 positive cells were detected, therefore a detailed stereological analysis was not appropriate and all positive cells were manually counted in each WD using an Axiolab microscope (Carl Zeiss Ltd, Hertfordshire, UK).

6.2.9 Studies on Testicular feminised (Tfm) mice

Testicular feminised (Tfm) mice were generously supplied by Prof. Peter O'Shaughnessy, for which I am extremely grateful (O'Shaughnessy and Murphy, 1993). Time-matings were set up in Glasgow University Vet School animal facility by Dr Paul Baker.

The Tfm colony is maintained as a balanced stock, meaning the Tabby (Ta) gene is on one X chromosome balanced against the Tfm gene on the other. Tfm mice were bred on a C3H/HeH-101/H genetic background from stock animals obtained from the MRC Radiobiology Unit Harwell. Matings were set up between Ta females that carry the Tfm deletion (XTa /XTfm) and sibling males (XTa/Y). This mating will produce Tabby males (XTa /Y), Tfm males (XTfm/Y), Tabby females (XTa / XTa) and Tabby females carrying the Tfm deletion (XTa /XTfm). The Tfm male mice do not carry the X-chromosome marker genes (i.e. Tabby) therefore if allowed to mature, the young produced with female external genitalia but with a normal coat are the XTfm/Y males, whereas females have a striped coat because they carry one copy of the Ta gene or are homozygous Tabby with their distinct phenotype (e.g. they have a waxy tail and bald patches behind the ears); XTa /Y males look similar to the homozygous Tabby females. However, this Tabby X-chromosome marker did not assist in differentiating between wild type female and Tfm male fetuses since fetal mice have no coat as yet. Instead, a genetic allelic discrimination test needs to be optimized to confirm fetal genotype. The appropriate controls used were normal males from the same genetic background.

6.2.9.1 Dissections

Dams were killed by cervical dislocation and fetuses were recovered at e16.5 and e18.5. Fetuses were killed by decapitation before reproductive tracts were recovered by microdissection and photographed using a Leica ICA camera (section 2.3.2.1). Reproductive tracts were analysed microscopically for any gross morphological abnormalities and WDs were fixed in Bouin's.

6.3 Results

6.3.1 Natural regression

6.3.1.1 Timing and pattern of natural regression of the female rat WD

Figure 6.2 shows images of gonads and MD and/or WDs from e16.5-18.5 fetuses. At e16.5, the female WD is still evident, lying medial to the MD. This WD begins to regress in a cranio-caudal direction and by e18.5, only the caudal portion of the female WD is evident. In the male, the WD remains a simple straight duct throughout this period with the male MD apparent at e16.5 but by e18.5, the MD cannot be identified when examined microscopically (Fig. 6.2).

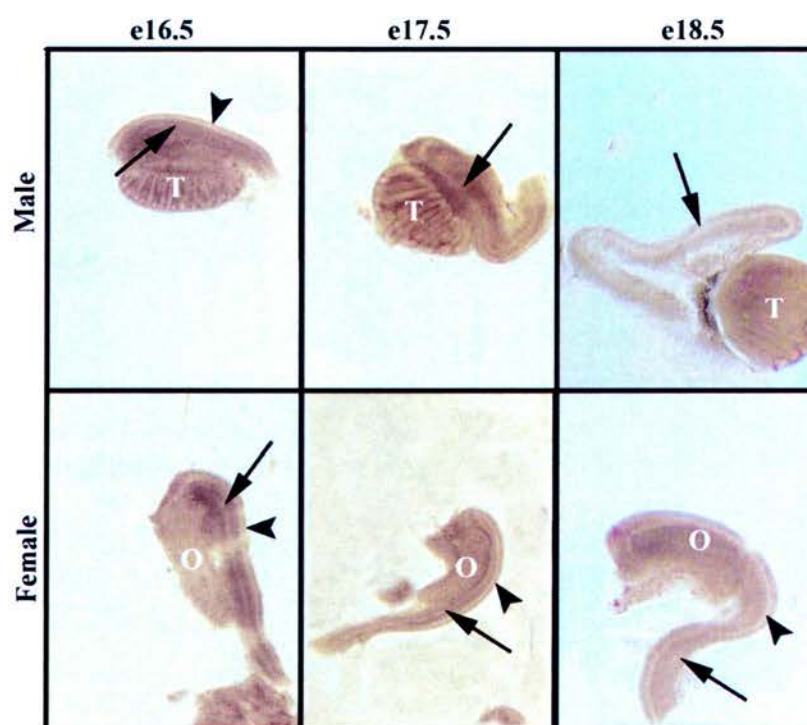


Figure 6.2 Representative reproductive tracts from e16.5-18.5 male and female rat fetuses highlighting the timing and pattern of Wolffian duct (WD) regression. Note that at e16.5, the female WD (arrow) is obvious, lying medial to the MD (MD, arrowhead). By e18.5, the female WD has almost completely regressed. Note also that the male MD is evident at e16.5 but has regressed by e17.5. All images are at the same magnification. T – testis O – ovary

6.3.1.2 Testosterone is not produced by the fetal rat ovary

Minimal testosterone levels were detected in the fetal ovary at e17.5, unlike the testis in which more than 17 fold higher levels were found (Fig. 6.3).

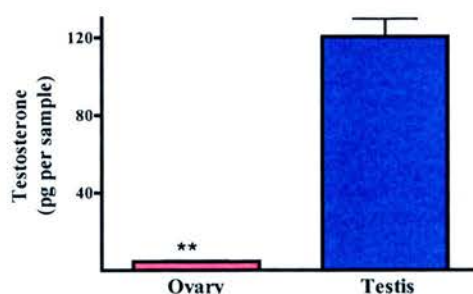


Figure 6.3 Testosterone (pg per sample) levels in the ovary and testis at e17.5. Values are mean \pm S.E.M for $n=3$. ** $p<0.01$, compared to testosterone levels in the testis

6.3.1.3 Androgen receptor is expressed in the regressing female rat Wolffian duct

Androgen receptor (AR) protein was detected by immunohistochemistry in both the male and female WD of rats at e17.5. In the male WD at e17.5, AR protein was intensely expressed in the stroma while the epithelium showed weaker immunoexpression (Fig. 6.4, panel A). In the e17.5 female WD, AR was detected in both the stroma and the epithelium: this was seen in all female WDs examined ($n=10$) (a representative WD is shown in panel B, Fig. 6.4).

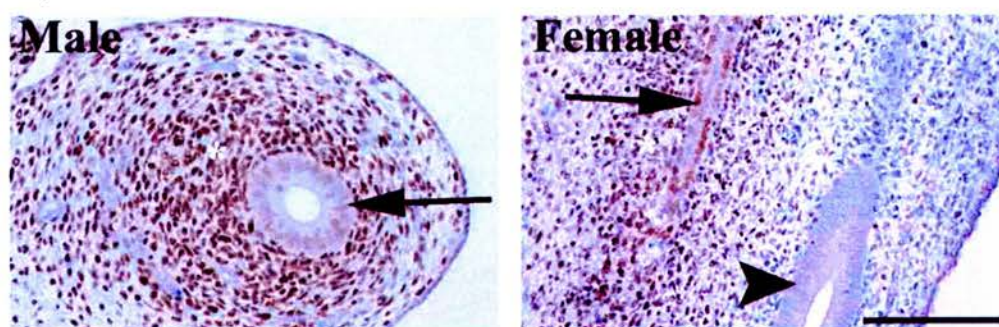


Figure 6.4 Immunoexpression of androgen receptor (AR) in the Wolffian duct (WD) in a male and female rat fetus at e17.5. Note that AR (brown) is strongly expressed in the stroma surrounding the male WD (arrow) but less intensely in the epithelium (A). Similarly, AR is expressed in both the stroma and epithelium in the female WD (B). Note that the female MD (arrowhead) is negative for AR protein by immunohistochemistry. Scale bar = 100 μ m

6.3.1.4 Role for apoptosis in the regression of the female rat WD

Apoptotic cells, indicated by cleaved caspase 3 positive staining, were evident in the epithelium of the female WD at e17.5 (Fig. 6.5). This is in contrast to the male WD from either control e17.5 or flutamide-exposed (100 mgkg⁻¹) e18.5 fetuses which were both negative for cleaved caspase 3 by immunohistochemistry (Fig. 6.5). Note in the male, the

remnant of the regressing MD could still be identified histologically at both e17.5 and e18.5 but no lumen was apparent.

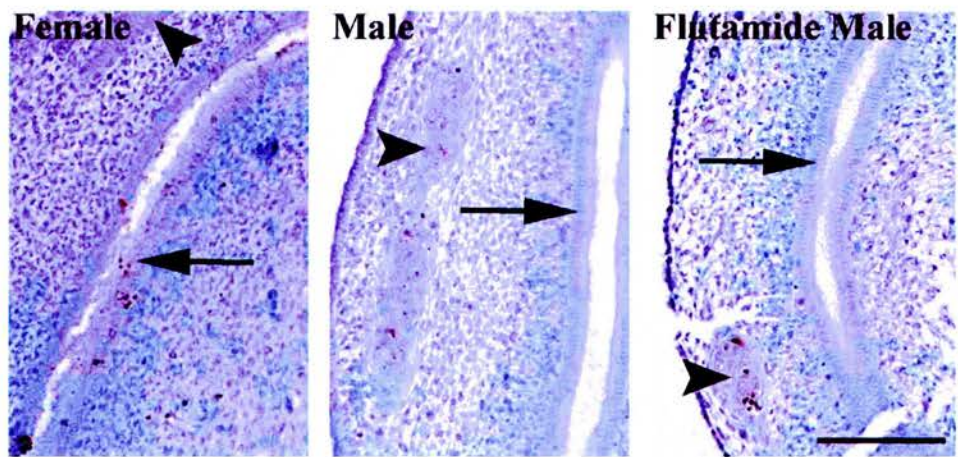


Figure 6.5 Frequency of apoptotic cells (immunopositive for cleaved caspase 3: brown staining) in representative Wolffian ducts (WDs) from control and flutamide-exposed (100 mgkg⁻¹) rat fetuses. Note that apoptotic cells were evident in the epithelium of the regressing female WD at e17.5 (arrow). Few apoptotic cells were seen in either male WDs (arrow) from control e17.5 or flutamide-exposed e18.5 fetuses. Note also that apoptotic cells were seen in the regressing MD (MD, arrowhead) in both control and flutamide-exposed male fetuses. Scale bar = 100 μ m.

6.3.1.5 Cell proliferation is absent in the regressing female rat Wolffian duct epithelium

Immunostaining for phospho-histone H3 showed mitotic cells were present in the stroma surrounding the regressing female WD at e18.0 but they were rarely detected in the epithelium (Fig. 6.6). This is in contrast to both control and flutamide-exposed male WDs in which mitotic cells were evident in the stromal and epithelial cell compartments (Fig. 6.6).

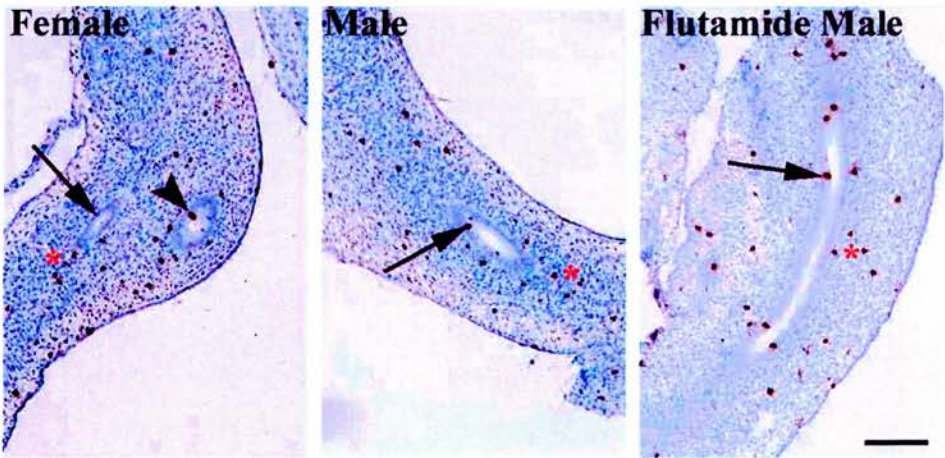


Figure 6.6 Frequency of mitotic cells (immunopositive for phospho-histone H3: brown staining) in Wolffian ducts (WD) from male and female fetuses at e18.0-18.5 in the rat. Note that cell mitosis was evident in the stroma surrounding the regressing female WD (A, e18.0) (*) but not in the epithelium (arrow) whereas mitotic cells were obvious in both the stroma (*) and epithelium (arrow) in control (B) and flutamide-exposed male fetuses at e18.5 (C, 100 mgkg^{-1}). Note also that mitotic cells were seen in the epithelium of the female MD (arrowhead, A). Scale bar = $100 \mu\text{m}$

6.3.1.6 Flattening of the epithelium in the regressing female rat Wolffian duct

Upon visual inspection, epithelial abnormalities were noted in the regressing female WD at all ages examined (e16.5-18.5), including an apparent reduction in epithelial cell height (this was not quantified) and a narrowing of the lumen, often appearing absent (Fig. 6.7). This is similar to the abnormalities seen in the male WD from fetuses exposed to flutamide (100 mgkg^{-1} ; see chapter 3). Note that in both the control and flutamide-exposed male, a remnant of the MD was apparent at e18.5, even though the MD could not be identified grossly at the time of dissection.

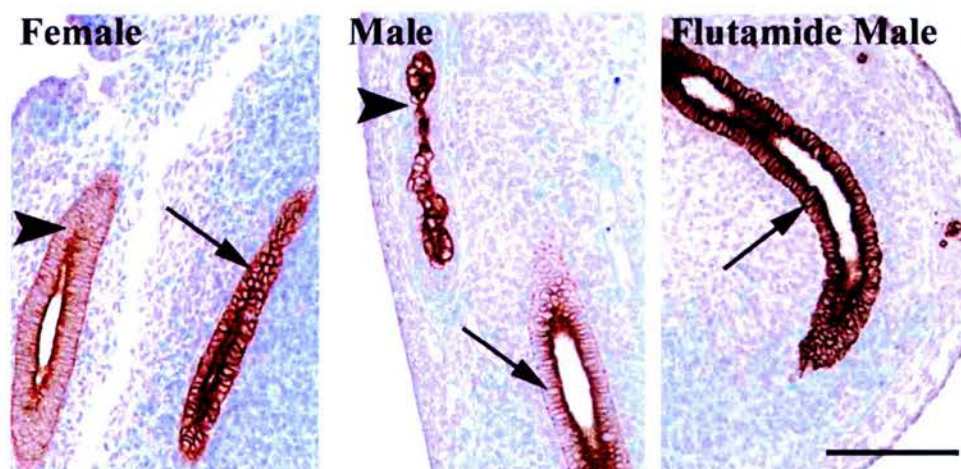


Figure 6.7 Representative abnormalities in the epithelium of Wolffian ducts (WD; immunostained for cytokeratin) from female and male fetuses at e18.0-18.5. Note the reduction in epithelial cell height and narrowing of the lumen in the regressing female WD at e18.0 (arrow) compared to the epithelium in control male WDs at e18.0 (arrow). This epithelial flattening was also noted in the epithelium in WDs from flutamide-exposed fetuses at e18.5 (arrow). Note also the reduction in epithelial cell height seen in the regressing MD (MD) in the male fetus at e18.0 (arrowhead) compared to the control female MD (arrowhead). Scale bar = $100 \mu\text{m}$

6.3.1.7 The basement membrane is interrupted in the regressing female Wolffian duct

Immunostaining for laminin highlighted that the basement membrane around the WD epithelium is interrupted and incomplete in the regressing female WD at e16.5 compared to the defined 'ring' of laminin seen in the basement membrane in control male WDs (Fig. 6.8). By e18.5, the female WD has almost completely regressed leaving patches of laminin staining where the epithelium was once present (Fig. 6.8).

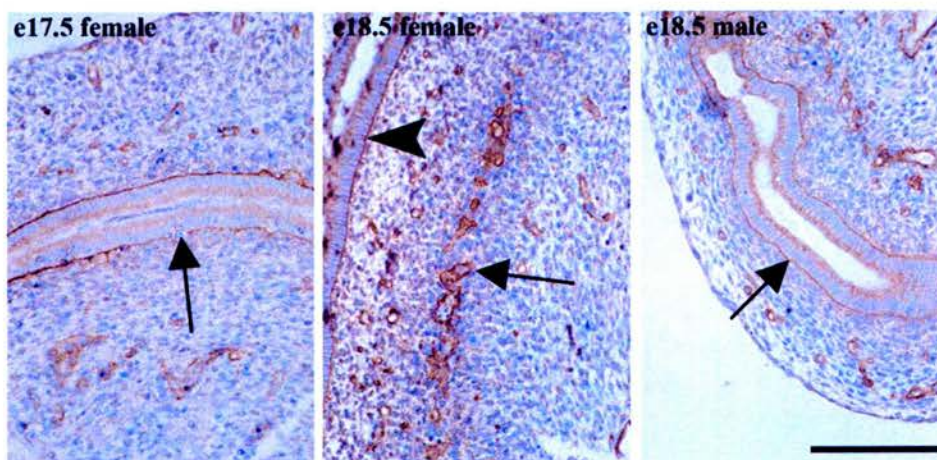


Figure 6.8 Demarcation of the basement membrane in Wolffian ducts (WD) from male and female fetuses at e16.5 and e18.5 in the rat. Note that laminin forms a defined 'ring' at the basement membrane in control male WDs (arrow, e18.5). In contrast, in the female WD at e16.5 (arrow) the basement membrane is interrupted in areas and by e18.5, the epithelium is absent leaving patches of laminin staining (B, arrow). Note also the presence of the female MD (arrowhead). All images are at the same magnification. Scale bar = 100 μm

6.3.1.8 Mesenchymal differentiation in the regressing female rat Wolffian duct

As previously shown, desmin protein was localised to the stroma surrounding the male fetal WD at all ages studied (e15.5-21.5; section 4.4.3.2). Desmin was also expressed in the stromal compartment surrounding the regressing female WD at all ages examined (e15.5-18.5) in a similar pattern to that seen in the male WD (Fig. 6.9). Desmin expression was not lost in the regressing female WD, even when the epithelium was flattened and the lumen was no longer evident (Fig. 6.9, panel B). Desmin was never expressed in the epithelium in either male or female WDs and was not detected in the stroma surrounding the MD in either males or females.

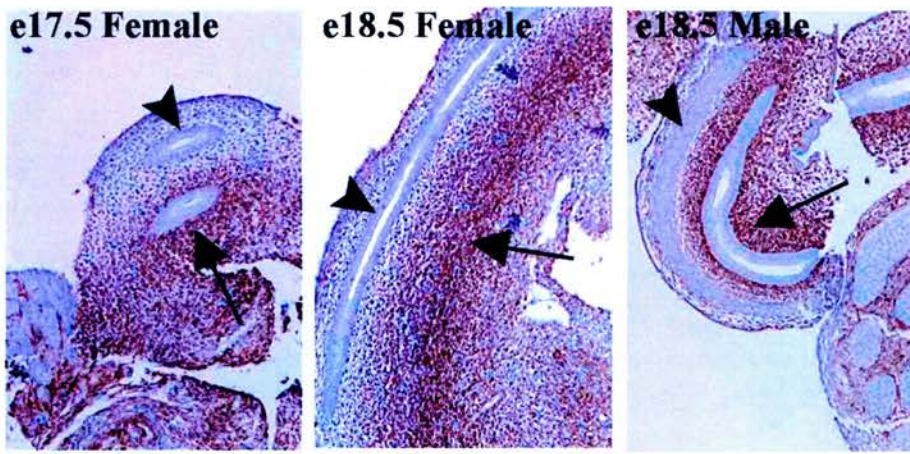


Figure 6.9 *Desmin protein expression in the male and female rat WD at e17.5 and e18.5. Note that desmin (immunostained brown) was detected in the stroma surrounding the female WD (arrow) at e17.5 and e18.5 as well as that surrounding the male WD at e18.5. Note also that desmin was not expressed in the stroma surrounding the MD (arrowhead) from either male or female fetuses.*

As reported in chapter 4, smooth muscle actin (SMA) was not expressed in the stromal compartment surrounding the male WD until e18.5 therefore SMA was not examined in the regressing female WD.

6.3.2 Wolffian duct regression in testicular feminised (Tfm) mice

In control mice, the female WD has almost completely regressed by e16.5 (Fig. 6.10) with only the cauda evident in some animals.

At e16.5, all WDs from male fetuses from a proven Tfm carrier dam looked morphologically similar (Fig. 6.10). By e18.5, there was an obvious morphological distinction between WDs from wild-type littermates, which were already considerably coiled by e18.5, and those from what were presumed to be Tfm males, which had no obvious WD with just a remnant, mesenchymal-like structure present with no epithelium or lumen (Fig. 6.10). Final confirmation of the genotype requires optimisation of the allelic discrimination assay.

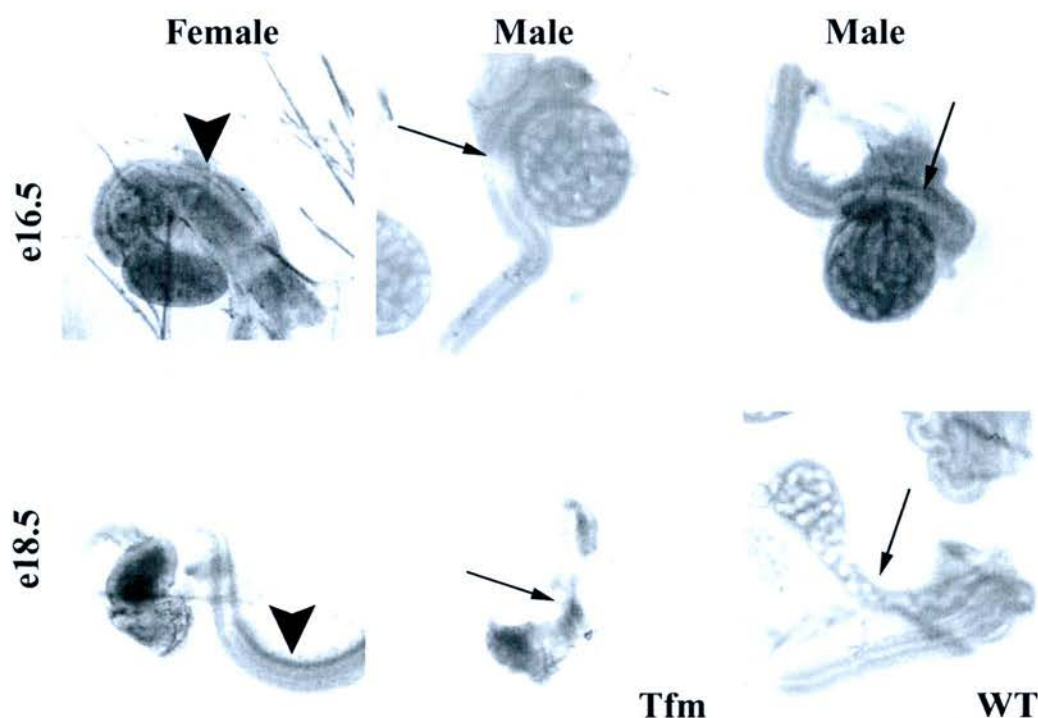


Figure 6.10 Representative Wolffian ducts (WD, arrow) from wild type (WT) and testicular feminised (Tfm) mice at e16.5 and e18.5. Note that at e16.5, the female WD has regressed and cannot be identified alongside the female Müllerian duct (arrowhead). At e16.5, intact WDs were identified in all male fetuses (top row) whereas by e18.5, Tfm male mice had only a remnant WD-like structure (bottom middle). In contrast, WT male WDs were notably coiled by e18.5 (bottom right).

6.3.3 Wolffian duct regression after more complete reduction of androgen action *In utero* in the rat.

6.3.3.1 Pattern of WD development after exposure to DBP + flutamide in the rat

At e18.5, the WD remains a simple straight duct in both control and flutamide-exposed (100 mgkg⁻¹) animals, as discussed in chapter 3. Combined exposure to DBP + flutamide did not result in any gross differences in the appearance of the male WDs at e18.5, compared to age matched control male WDs (Fig. 6.11).

By e21.5, control male WDs were highly coiled and this was reduced by exposure to either flutamide (100 mgkg⁻¹) or DBP (500 mgkg⁻¹) alone (Fig. 6.11 and Fig. 6.12). As well as the reduction in coiling, some WDs from flutamide- or DBP-exposed fetuses appeared incomplete at e21.5 (Table 6.2); this was never seen at e18.5 or in control WDs and affected WDs always had a short lumen apparent at the caput end. As detailed in chapter 3, the

incidence of incomplete WDs increased at around puberty in flutamide-exposed animals resulting in the majority of adults having no WD-derived tissues. In contrast, the incidence of incomplete WDs remained relatively constant in postnatal animals after fetal exposure to DBP alone (Table 6.2).

Further reducing androgen action by combining exposure to DBP + flutamide resulted in an almost complete loss of any WD structure in all fetuses examined at e21.5: a lumen was only apparent in the caput segment of the WD, proximal to the efferent ducts (Fig. 6.11 and Table 6.2). This further reduction in luminal length was quantified, as shown in Fig. 6.12: it is worth noting that since a lumen was only apparent at the caput in e21.5 WDs exposed to DBP + flutamide, these luminal length measurements represent only this short lumen.

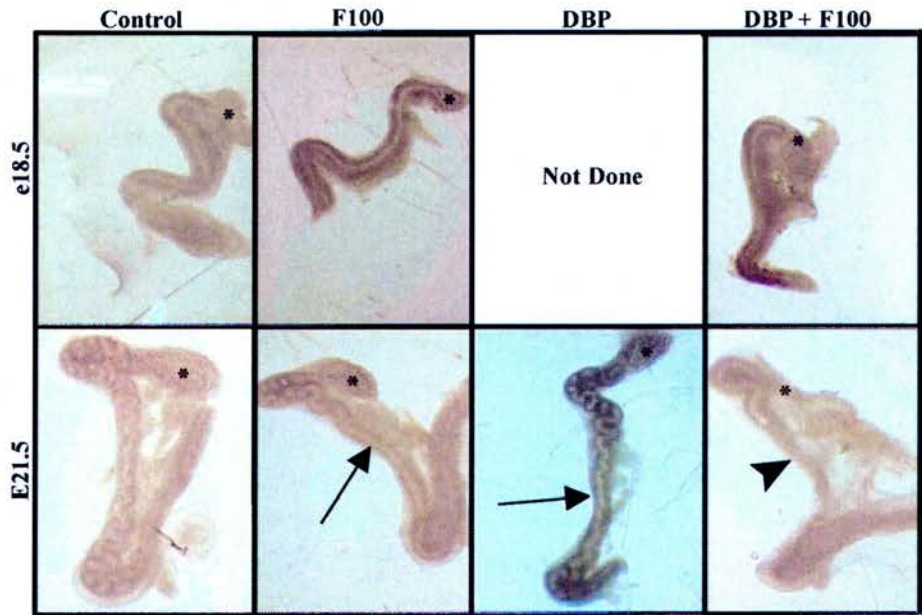


Figure 6.11 Representative Wolffian ducts (WD) in males from control, flutamide-exposed (100 mgkg^{-1}), DBP-exposed (500 mgkg^{-1}) or DBP + flutamide-exposed litters at e18.5 and e21.5. Note that at e18.5, all WDs were uncoiled but were complete, even after exposure to flutamide or DBP + flutamide. By e21.5, control WDs were highly coiled. Note that this coiling was reduced after exposure to flutamide or DBP alone (arrow). Note that exposure to DBP + flutamide together resulted in severe abnormalities in the WD at e21.5 with a loss of normal structure and no lumen apparent distal to the caput (arrowhead). All images are at the same magnification. * - efferent ducts

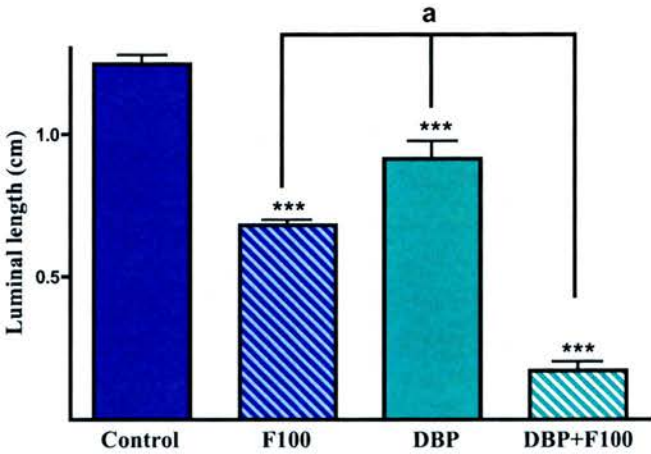


Figure 6.12 Quantification of coiling (= luminal length) of e21.5 WDs from control and treated litters. Note the significant reduction in luminal length in WDs from animals exposed in utero to 100 mg/kg-1 flutamide (blue striped bar), DBP (solid green bar), or DBP + flutamide (green striped bar), when compared to controls. Note also that exposure to DBP reduced luminal length less than did flutamide-exposure while exposure to DBP + flutamide decreased luminal length even more than flutamide alone. Values are mean \pm S.E.M for $n=12-66$ WDs, *** $p<0.001$, in comparison to the respective control value, a $p<0.001$ compared to F100 value.

Table 6.2 Percentage of rats with an intact Wolffian duct (WD, e18.5 and e21.5) or WD-derived tissues (Pnd17 and adult) after treatment-induced impairment of androgen action in fetal life.

	Control	Flutamide 100	DBP	DBP + F100
e18.5	100% ⁴	100%	N/D ⁵	100%
e21.5	100%	89%	86%	0%
Pnd 17	100%	87%	83%	0%
Adult	100%	17%	83%	0%

2

⁴ Note that 100% means that all WDs, or its derivatives, were complete while 0% means no complete WDs were seen.

⁵ N/D – studies not yet done

6.3.3.2 Androgen receptor expression in rat WDs after exposure to DBP + flutamide

Androgen receptor (AR) protein was immunoexpressed intensely in the stroma surrounding the WD but less intensely in the epithelium at e18.5 (Fig. 6.13). By e21.5, AR was intensely immunoexpressed in both the epithelium and stroma (Fig. 6.13). AR protein expression was not obviously altered by exposure to flutamide (Fig. 6.13 and section 4.4.1.1). Combined exposure to DBP (500 mgkg^{-1}) + flutamide (100 mgkg^{-1}) did not obviously alter immunoexpression of AR in the stromal compartment but AR was not detectable in the epithelium at either e18.5 or e21.5 (Fig. 6.13). Exposure to DBP alone did not have any obvious impact on AR expression in the WD (data not shown).

Note that at e21.5, little epithelium was observed in the WD from fetuses exposed to DBP + flutamide with any persistent epithelium only identifiable in the caput region. In order to highlight the effects of DBP + flutamide treatment on the epithelium, several WDs had to be examined to identify any residual epithelium with many WDs showing no sign of any obvious epithelium.

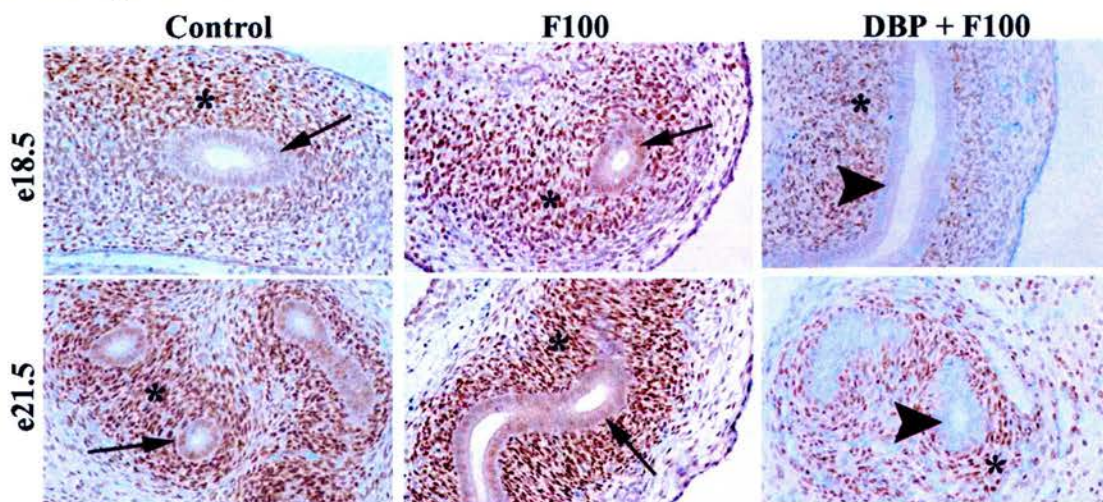


Figure 6.13 *Immunohistochemical expression of androgen receptor (AR) in representative rat Wolffian ducts (WD) at e18.5 and e21.5. Note that AR protein is expressed in the stroma (*) and epithelium (arrow) at e18.5 and e21.5 in both control and flutamide-exposed (100 mgkg^{-1}) fetuses. Combined exposure to DBP (500 mgkg^{-1}) + flutamide (100 mgkg^{-1}) did not alter AR expression in the stroma (*) but prevented AR expression in the epithelium (arrowhead) at both e18.5 and e21.5. All images are at the same magnification.*

6.3.3.3 The role for apoptosis in the rat Wolffian duct after exposure to DBP + flutamide

Apoptosis was not apparent in WDs from control, flutamide-exposed (100 mg kg^{-1}) or DBP + flutamide-exposed fetuses at e21.5 (data not shown). It is worth noting that by e21.5, little epithelium was present in WDs exposed to DBP + flutamide together.

6.3.3.4 The presence of mitotic cells in the rat Wolffian duct after exposure to DBP + flutamide

Cell mitosis was evident in both the stromal and epithelial cell compartments of WDs from control and DBP + flutamide-exposed animals at all ages (Fig. 6.14). Immunostaining for phospho-histone H3 suggested fewer mitotic cells were present in WDs from DBP + flutamide-exposed fetuses compared to controls (Fig. 6.14) but this was not confirmed quantitatively. This was particularly evident in the epithelium where mitotic cells were rarely noted in WDs exposed to DBP + flutamide (Fig. 6.14). Note that in WDs from males exposed to DBP + flutamide epithelium was only seen in the caput region.

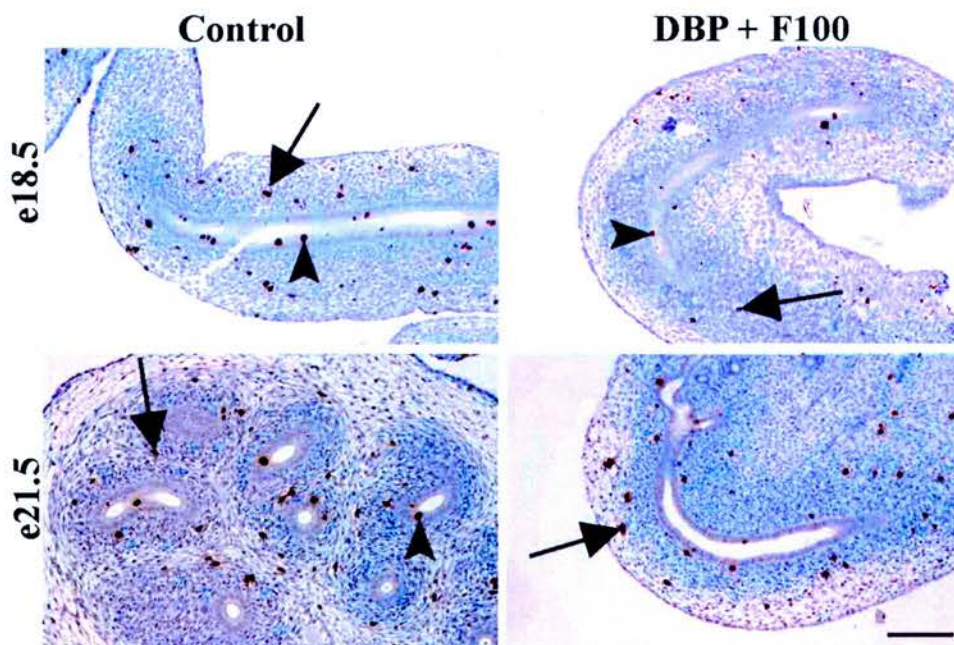


Figure 6.14 Frequency of mitotic cells (immunopositive for phospho-histone H3; brown staining) in representative Wolffian ducts (WDs) at e18.5 and e21.5 from control and DBP + flutamide- exposed fetuses. Note the numerous mitotic cells in the stroma (arrow) and epithelium (arrowhead) in control WDs. There is an apparent reduction in the number of mitotic cells in WDs from DBP + flutamide-exposed animals. Scale bar = 100 μm

6.3.3.5 Impact of combined exposure to DBP + flutamide on the rat WD epithelium

Histological examination of WDs revealed no obvious histological differences between WDs from control and flutamide-exposed fetuses at e18.5 (data not shown). However, as seen in Fig. 6.15, combined exposure to DBP + flutamide resulted in flattening of the epithelium, even at e18.5. The extent of this flattening varied between WDs: Fig. 6.15 shows examples of WDs with relatively unaffected epithelium and WDs with significant collapse of the epithelium. This flattening was not seen in controls or flutamide-exposed WDs at this age. By e21.5, epithelial abnormalities were detected in WDs from fetuses exposed to flutamide (100 mg kg^{-1}), including flattening or even loss of epithelium lining the lumen and widening or narrowing of the lumen (see Fig. 3.20). These abnormalities were again noted in any persistent epithelium in WDs from fetuses exposed to DBP + flutamide: the majority of such animals had very little epithelium evident, especially in regions distal to the caput and any epithelium present was greatly flattened with an abnormal lumen (Fig. 6.15). Note that the images depicted in Fig. 6.15 show rare persistent areas of epithelium in WDs from these animals and these were only detected in the caput region of the WD from a few fetuses.

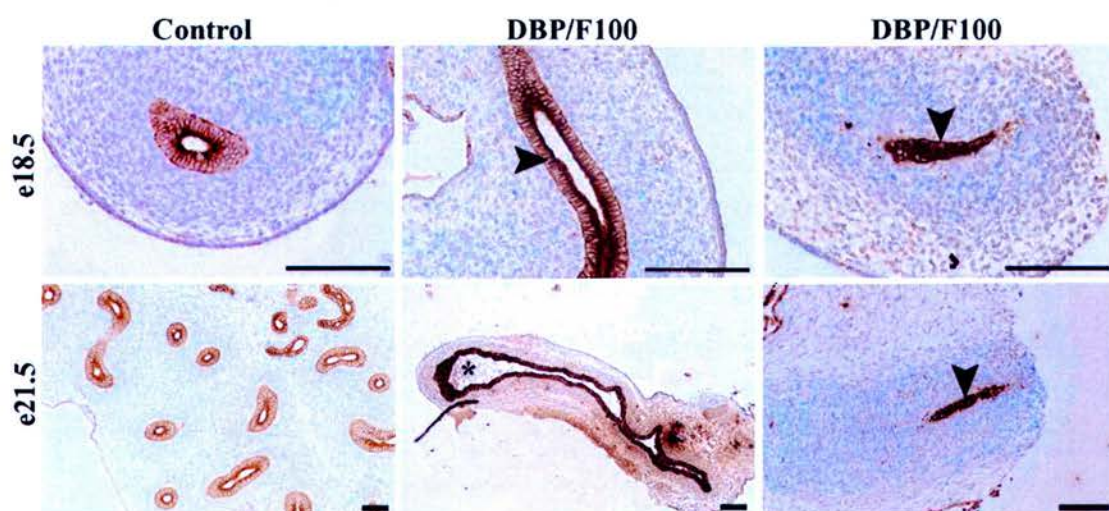


Figure 6.15 Representative abnormalities in epithelia (immunostained for cytokeratin) of rat Wolffian ducts (WD) from e18.5 and e21.5 fetuses. Note the reduction in epithelial cell height seen after exposure to DBP (500 mg kg^{-1}) + flutamide (100 mg kg^{-1}) at both e18.5 and e21.5 (arrowhead). Note also the widened lumen seen in some WDs (*) compared to the flattened lumen in others. Scale bars = $100 \mu\text{m}$

6.3.3.6 Combined exposure to DBP + flutamide interrupts the basement membrane

Laminin was immunolocalised to the basement membrane in WDs at all ages studied and at e21.5, this laminin expression was interrupted by exposure to flutamide (100 mg kg^{-1}) (see

section 4.4.4.1). Exposure to DBP + flutamide together did not interrupt laminin expression in the basement membrane at e18.5 but by e21.5, laminin expression was less defined and was often absent (Fig. 6.16).

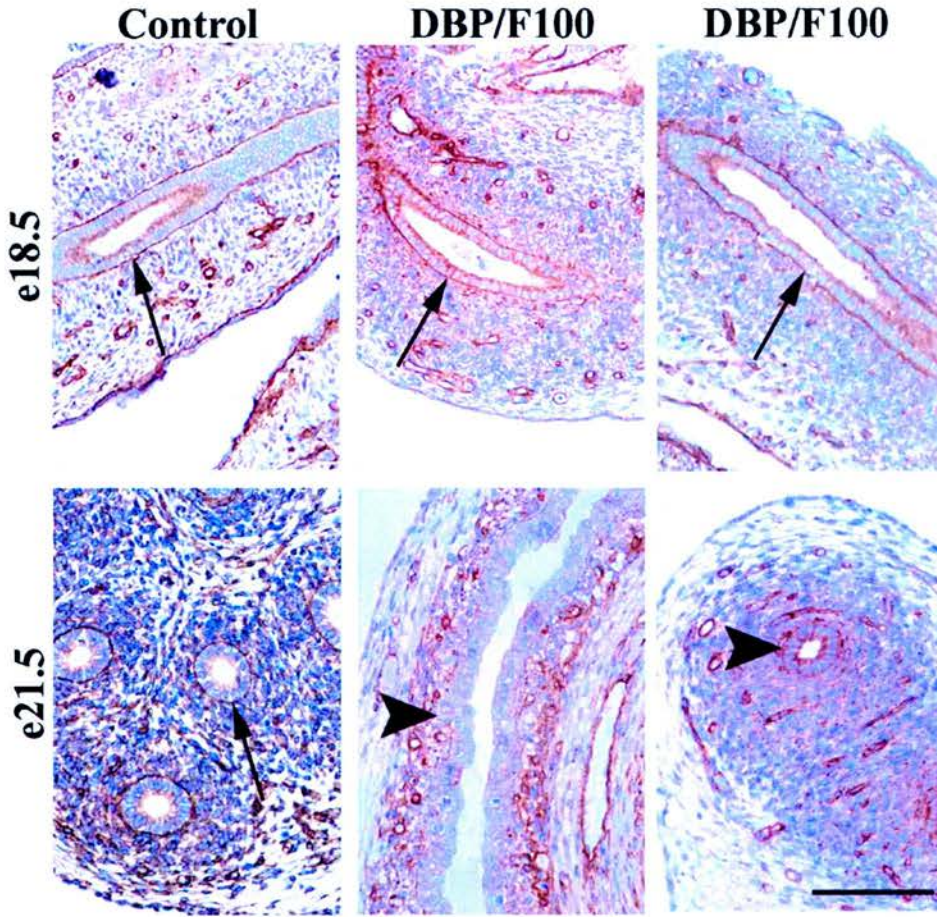


Figure 6.16 Laminin protein expression (immunostained brown) in the basement membrane in WDs at e18.5 and e21.5. Note the defined 'ring' of laminin around the base of the epithelium in control WDs at e18.5 and e21.5 (arrow). Exposure to DBP + flutamide did not alter this laminin expression at e18.5 (arrow) but by e21.5, combined exposure to DBP + flutamide resulted in interrupted laminin expression (arrowhead). Scale bar = 100 μ m.

6.4 Discussion

In females, the WD naturally regresses during fetal life: this is believed to be due to a lack of androgens (Huhtaniemi, 1994). However, little is known about the timing of or mechanisms controlling WD regression in female rats. In our rat colony, a full length WD can still be identified in female fetuses at e16.5, lying medial to the developing MD and lateral to the ovary. This WD begins to regress between e16.5 and e18.5 in a cranio-caudal direction with only the caudal segment still evident by e18.5. These timings agree with those previously published (Stinnakre, 1975; Inomata *et al*, 1989; Jirsova and Vernerova, 1993). In vitro testosterone assays confirmed that at e17.5, testosterone was present in the fetal testis but was barely detectable in the ovary at e17.5. This agrees with previous studies which have shown that throughout fetal life, plasma testosterone levels are at least 4 time higher in male fetuses than in females, where plasma testosterone levels remained very low (Habert and Picon, 1984). Conversely, androgen receptor (AR) protein was detected in the female WD at all ages studied, indicating that it is capable of responding to androgens if they were available. This highlights the bipotential fate of the WD in males and females.

Histological analysis revealed that female WD regression is, at least partially, due to both apoptosis and a lack of proliferation in the regressing epithelium. This is in contrast to findings in male WDs from rats exposed to flutamide *in utero*, in which apoptosis was not evident and mitotic cells were still identified in the WD epithelium (discussed in chapter 3). This observation of apoptosis in the regressing female WD is in agreement with previous studies (Dyche, 1979; Jirsova and Vernerova, 1993; Roberts *et al*, 1999; Allard *et al*, 2000) and suggests that the WD is undergoing active cell death in the female rather than simply not differentiating as appears to be the case in flutamide-exposed WDs (see chapter 3). Contrastingly, mitotic cells were evident in the stromal compartment of the regressing female WD at all ages studied (e16.5-18.5), even when the epithelium was no longer apparent. It has been suggested that the mesenchymal cells from the regressing male MD may migrate and thereafter contribute to the mesonephros (Austin, 1995): a similar phenomenon may occur in the regressing WD and might explain the persistence of cell mitosis during WD regression. The present findings highlight the differences in the cellular mechanisms involved in the natural regression of the female WD compared to those which result in the abnormalities seen in flutamide-exposed male WDs. These differences are likely to be due to differences in the level of androgen blockade with females having no androgen action while flutamide, at the doses used in these studies, is unlikely to cause a

complete blockade of androgen action and the residual low levels of androgen action appear to be sufficient to prevent WD regression.

Apoptosis was evident in the regressing rat WD and MD, as previously shown by Donohoe *et al* (Price *et al*, 1977). However, it has been suggested that apoptosis alone is not sufficient to cause regression of the MD, epithelial-mesenchymal transformation is also reported to be involved (Xavier and Allard, 2003). By e17.5, abnormalities in both gross morphology and histology were evident in the regressing female WD, including flattening of epithelial cells, narrowing and subsequent disappearance of the lumen, and interruption and loss of the basement membrane. These findings agree with previously published studies investigating WD (Jirsova and Vernerova, 1993) and MD (Dyche, 1979; Inomata *et al*, 1989; Austin, 1995; Magro and Grasso, 1995) regression. It has been suggested in the MD that the loss of basement membrane allows epithelial cells to protrude through into the mesenchymal compartment and de-differentiate into mesenchymal-like cells (Trelstad *et al*, 1982). These abnormalities mirror those noted in the flutamide-exposed WD at e21.5 (detailed in chapter 4) and suggest that during female WD regression, epithelial cells lose their attachment to the basement membrane and lose their polarity. It has been suggested that these changes may even be the trigger for these cells to enter apoptosis (Xavier and Allard, 2003).

Smooth muscle actin (SMA), an androgen responsive stromal differentiation marker (Schlatt *et al*, 1993), was not detected in the stroma of the male WD until e18.5 (section 4.4.2.2.1) and, therefore was not available as a marker for studying stromal development in the regressing female WD. Desmin, one of the earliest markers of mesenchymal differentiation into muscle, was detected in the male WD stroma at all ages studied (e15.5-e21.5). Since intermediate filaments are required not only to support cells but are also involved in signal transduction from the cell membrane to the nucleus, it was hypothesised that desmin may play a role in signal transduction in the developing WD (Alberts, 1994). Desmin was expressed in the regressing female WD at all ages studied (e16.5-18.5), even in areas where no epithelium or lumen was evident. This is consistent with findings in male WDs, in which flutamide did not induce a reduction in desmin expression. This suggests that the mesenchymal differentiation into muscle is at least partially independent of androgen action in both male and female WDs.

Studies in the female fetal rat have therefore confirmed that, in the absence of androgens the WD regresses and has almost entirely degenerated by e18.5. In order to further investigate WD regression, pregnant rat dams were exposed to both Di-n-butyl phthalate (DBP; 500 mgkg⁻¹), another anti-androgenic compound with a different mechanism of action than flutamide. DBP reduces fetal testicular testosterone production by 70-90% (Fisher *et al*,

2003; Lehmann *et al*, 2004) and results in impaired WD coiling at e21.5 in a similar manner to that observed in flutamide-exposed animals (discussed in chapter 3). This phenotype is consistent with previous studies (Barlow and Foster, 2003; Bowman *et al*, 2005). It is assumed that this reduction in WD coiling is due to the decrease in testicular testosterone production however it cannot be ruled out that DBP could directly affect the WD, independent of testosterone action. Coiling was reduced to a lesser degree by DBP exposure than by exposure to flutamide (100 mgkg^{-1}): this difference is likely to be due to the different mechanisms of action by each compound. It is likely that the fetal testis produces testosterone in excess of the levels required for masculinisation, with the result that DBP-induced reduction in testosterone production may not reduce the levels of androgen action as greatly as exposure to flutamide does. Flutamide prevents testosterone binding to the AR and since there are a limited number of cells expressing AR in the developing WD, this mechanism is likely to have a greater impact on androgen action than interfering with testosterone production, which is not in such a limited supply.

Like flutamide exposure (discussed in chapter 3), exposure to DBP also resulted in incomplete WDs with segments of the corpus missing in 14% of animals studied at e21.5. However, contrary to the increased incidence noted in flutamide-exposed animals after puberty, the incidence of missing segments remained relatively constant in DBP-exposed animals at later ages. The increased prevalence of epididymal abnormalities noted in animals exposed to flutamide *in utero* was hypothesised to be due to a failure to establish normal patterning of the WD in the fetus thus resulting in irrecoverable malformation of the reproductive tract and likely impairment of fertility. However, it is likely that the low level of testosterone still available after DBP-exposure is sufficient to activate AR dependent gene expression and thereby induce androgen-dependent WD patterning. The abnormalities reported here are in agreement with previous studies showing that DBP exposure results in a high incidence of epididymal abnormalities (Mylchreest *et al*, 1998; Mylchreest *et al*, 1999; Foster *et al*, 2000; Mylchreest *et al*, 2000; Foster *et al*, 2001; Barlow and Foster, 2003; Bowman *et al*, 2005).

In order to investigate WD regression further, an *in vitro* model was sought to allow further reduction of androgen action than exposure to flutamide or DBP alone, since neither induced WD regression as occurs naturally in the female. Pregnant rats were therefore exposed to both DBP (500 mgkg^{-1}) and flutamide (100 mgkg^{-1}) in combination (DBP + F100), resulting in a more complete blockade of androgen action. At e18.5, WDs from all DBP + F100-exposed animals were morphologically intact, suggesting that AR-mediated signalling was still not completely blocked fetally, thus allowing the WD to be rescued. In contrast, by

e21.5 the male WD had almost entirely regressed in all DBP + F100 fetuses leaving only a small segment of epithelium at the caput. It is likely that this region survives as it is the region that is closest to the testis and therefore will benefit from delivery of testosterone down the WD lumen. It was surprising that the WD appeared normal at e18.5, an age when the female WD has almost entirely regressed, but had almost completely degenerated by e21.5.

In order to investigate this phenomenon further, studies were undertaken in testicular feminised (Tfm) mice, which allowed study of the effects of complete blockade of androgen action on male WD development (Gehring and Tomkins, 1974; Murphy and O'Shaughnessy, 1991). These mice also exhibit reduced serum testosterone levels in adulthood as they are known to be deficient in 17 alpha-hydroxylase, a key enzyme in steroidogenesis (Murphy and O'Shaughnessy, 1991; Jones *et al*, 2003): fetal testosterone levels have not been reported, however, fetal Leydig cells appear normal (personal communication with Prof. Peter O'Shaughnessy). Previously, identification of adult Tfm male rats has relied upon coat colour and the absence of any WD-derived tissues, therefore a genotyping assay is required to identify Tfm fetal males. I am currently optimising this assay, which will entail allelic discrimination using Taqman PCR and this will be used to confirm the findings reported in this thesis. Gross morphological analysis revealed that at e16.5, all male littermates had intact but relatively uncoiled WDs. This is in contrast to the normal female mouse in which the WD has almost completely regressed by e16.5, with a lumen only evident at the caudal end. These observations are in agreement with timings previously published (Tsuji *et al*, 1992; Staack *et al*, 2003). By e18.5, within one litter WDs from some males are considerably convoluted whereas other males had only a WD remnant with no obvious lumen or normal structure: these are presumed to be Tfm males but this requires confirmation. It is not immediately clear why the WD would regress slower in these Tfm males, which are devoid of a functional androgen receptor (He *et al*, 1991; Murphy and O'Shaughnessy, 1991), than in normal females. Proven Tfm carrier dams were used in the matings therefore at least some of the male offspring should be Tfm.

These findings in Tfm mice and DBP + F100 exposed rats raise the question of whether, in the absence of high levels of androgen action, some other mechanism may be trying to compensate to rescue the fetal male WD. If so, it would seem that this mechanism alone is not sufficient to maintain the WD long term since, in both models the male WD ultimately undergoes degeneration before birth. Possible candidates involved in this compensatory mechanism are oestrogens, inhibins and/or Insulin-like factor (Insl3). Insl3 is believed to play a role in testis descent (Nef and Parada, 1999; Emmen *et al*, 2000; Kumagai *et al*, 2002;

McKinnell *et al*, 2005) and *Ins13* and its receptor, *LGR8*, have been shown to be expressed in the developing WD (unpublished data and personal communication with Bernard Jegou). *Ins13* knockout mice show normal WD development (Adham *et al*, 2000), however these mice have normal androgen action, therefore it is unknown what effect a lack of both androgen and *Ins13* action would have on the developing WD: this phenomenon requires further investigation.

Tfm mice are the mouse equivalent of complete androgen insensitivity syndrome (CAIS). CAIS patients are XY males but with female external genitalia (Tsuji *et al*, 1991; Ahmed *et al*, 2000; Brinkmann, 2001; McPhaul, 2002; Quigley, 2002). Most evidence from CAIS patients is postnatal (Hannema *et al*, 2004) with no definitive evidence published detailing the status of the human fetal WD. It is therefore unclear whether in CAIS patients the WD fails to stabilise completely during fetal life, as occurs normally in females, or whether the absence of WD structures in later life results from 'post-differentiation' degeneration of WD-derived tissues, as occurs in rats exposed *in utero* to flutamide. These preliminary findings in male Tfm mice support the latter view but require further investigation.

The androgen receptor (AR) is normally expressed in the WD epithelium and surrounding stroma by e18.5 and this expression pattern is not altered by exposure to flutamide (detailed in chapter 4). AR was expressed normally in the stromal compartment in animals exposed to DBP + F100 but was not detected in the epithelium at either e18.5 or e21.5. This lack of epithelial AR expression may explain the degeneration of the WD by e21.5 as, in other systems, epithelial AR is believed to be vital for tissue morphogenesis (Lang *et al*, 2001; Cunha *et al*, 2004). These results also suggest that epithelial AR expression is dependent on AR signalling from the stroma whereas stromal AR expression appears to be either dependent on much lower levels or even independent of androgen action. However, in the regressing female WD, AR can be detected in both the stroma and the epithelium raising the question of what controls epithelial AR expression and whether AR expression and/or WD set up are different in the male and female: this again brings into question the long standing belief that the decision for the WD to regress or develop is solely dependent on androgen action.

Exposure to DBP + F100 resulted in similar histological abnormalities at e21.5 as those observed in both the regressing female WD and in the flutamide-exposed male WD, including flattening of the epithelium, narrowing and loss of lumen and interrupted basement membrane. These observations suggest that a similar mechanism is operating in each of these models whereby androgen action is reduced compared to age matched male controls. However, these abnormalities were not noted at e18.5, again suggesting that exposure to

DBP + F100 does not interfere with WD stabilisation at e18.5 but results in later degeneration by e21.5. This further highlights the possibility for a role for another compensatory mechanism in the early male WD if androgen action is low.

As in both the flutamide-exposed male and the naturally regressing female WD, the most obvious explanations for the loss of WD tissue in DBP + F100 males are an increase in cell apoptosis and/or a decrease in cell mitosis. Mitotic cells were evident in DBP + F100-exposed WDs at both e18.5 and e21.5, however, the number of mitotic cells appeared to be reduced compared to age matched control WDs, especially in the small remnant of epithelium. This reduction was more apparent in the corpus and cauda than in the caput where a lumen and epithelium were still evident. This suggests that reduced cell proliferation may contribute to the loss of WD tissue after exposure to DBP + F100. On the other hand, cleaved caspase 3 positive cells were rarely observed in these WDs at either e18.5 or e21.5. This suggests that apoptosis was not involved, however, at e18.5 the WD looks normal and by e21.5 the majority of the WD has already degenerated thus suggesting we are studying the wrong time points. It is possible therefore that apoptotic cells would be seen in DBP+F100 WDs at e19.5 or e20.5, especially since apoptotic bodies could be identified in the degenerating WD at e21.5.

6.5 Conclusion

It can be seen from these studies that the female WD regresses in rats between e16.5-e18.5. This regression is due to both apoptosis and a lack of proliferation in the WD epithelium. This is in contrast to male WDs from fetuses exposed to flutamide *in utero* in which apoptosis was not detected and proliferation is still apparent in both the epithelium and the stroma, but at a reduced level compared to age matched controls. This again highlights that flutamide does not induce regression of the male WD but prevents its subsequent differentiation. Disruption to the ECM and basement membrane were evident in both the naturally regressing female WD and in flutamide-exposed male WDs; it cannot easily be distinguished whether these disruptions induce the abnormalities seen in the WD in both these models or if they occur as a result of the disturbed WD development.

Studies in both Tfm mice and rats exposed to DBP and flutamide in combination (DBP + F100) again highlight the essential role for high levels of androgen action in WD stabilisation and development; however, since the early WD does not regress at the same age as occurs naturally in the female, this raises the question of whether another factor may be trying to compensate for the great reduction in or absence of androgen action. This phenomenon warrants further investigation.

7 Rescue of the female WD by exogenous testosterone exposure

7.1 Introduction

As discussed in chapter 6, regression of the female rat WD occurs between e16.5-18.5 due to a lack of androgens. However, the studies in Tfm mice and rats exposed to DBP + flutamide in combination raised the question of whether androgens alone were acting to stabilise the male WD or whether some other compensatory factor may play a role in rescuing the male WD. The experiments detailed in this chapter will address this question and examine whether exposure to exogenous androgens during gestation is sufficient to rescue the female WD.

Evidence from clinical observations suggests that the female fetus is vulnerable to masculinisation by androgen exposure (discussed in section 1.5.3.1). For example, fetuses with congenital adrenal hyperplasia (CAH) have an increased production of adrenal testosterone resulting in masculinisation of the external genitalia in the female fetus (reviewed in Merke and Bornstein, 2005). However, it is worth noting that these patients do not tend to have masculinised WD-derived structures: this is likely to be due to a lack of sufficient levels of local androgens as these patients are exposed to systemic androgens (section 1.5.3.1).

The ability of androgens to masculinise the female fetus is further substantiated by evidence gained from experiments in which animals have been exposed to exogenous androgens during fetal life (section 1.5.3.2). Exposure to exogenous androgens during gestation can elicit varying degrees of masculinisation in the female offspring, depending on the level and timing of exposure (Swanson and Werff ten Bosch, 1965; Ogawa and Nozawa, 1969; Jost *et al*, 1973; Stinnakre, 1975; Wolf *et al*, 2002; Wolf *et al*, 2004). This is not surprising since the regressing female UGT expresses ARs, highlighting its capability to respond to androgens (Chapter 6; Bentvelsen *et al*, 1995). The timing of testosterone exposure is critical for masculinisation of female fetuses: for example, in order to rescue the female WD, testosterone exposure must commence prior to e18 in rats, by which time the female WD has committed to regress (Swanson and Werff ten Bosch, 1965; Ogawa and Nozawa, 1969; Stinnakre, 1975). Previous studies have suggested that the female external genitalia are the most sensitive structures to the effects of testosterone with rescue and differentiation of the female WD requiring higher levels of testosterone (Greene, 1939; Swanson and Werff ten Bosch, 1965; Ogawa and Nozawa, 1969; Stinnakre, 1975; Wolf *et al*, 2002). However, considerable variation can be seen in the dose of testosterone exposure required to rescue and differentiate the female WD: Wolf and colleagues (2002) were unable to rescue the WD

in female Sprague-Dawley rats, even with 10 mg/rat testosterone propionate between e14-19 (total dose 60 mg) whereas this dose caused masculinisation of the external genitalia. However, Greene and colleagues (1939) noted that exposure to high doses of testosterone (80-90 mg total dose) in Wistar rats was not only able to completely rescue the WD but could stimulate some degree of coiling of the epididymis while lower testosterone doses (13.5 – 40 mg total dose) could often only rescue the WD unilaterally with the adult derived structures were often incomplete and lacked patent lumens. Unfortunately, the phenotype of these rescued WDs is merely described without any quantitative data or images, so that direct comparisons cannot be made with the normal male WD-derivatives. Further investigation is therefore required to clarify the dose and timing of testosterone exposure required to masculinise the female WD and the degree of differentiation that can be initiated.

7.1.1 Aims

To examine the impact of exogenous gestational testosterone exposure on fetal development.

To investigate if exogenous gestational testosterone exposure is sufficient to rescue and differentiate the female WD in rats.

To investigate if testosterone acts during the same critical window to rescue the female WD as it does to stabilise the male WD, as discussed in chapter 5.

To investigate if concomitant flutamide exposure can block the effects of exogenous testosterone exposure.

7.2 Methods

7.2.1 *In utero* treatments

As detailed in section 2.2, pregnant dams were randomly allocated to the various treatment groups detailed below and dosed once daily according to maternal bodyweight between e14.5-e21.5, or until the day prior to cull if this was before e21.5. This window of exposure was selected to begin just before the onset of endogenous androgen production in fetal males at e15.5 and the period of fetal male reproductive development (e15.5-21.5) according to Warren *et al* (1972). The dose of flutamide was selected based on results previously reported in chapters 3 and 4 highlighting that fetal exposure to flutamide induced reproductive tract abnormalities in both fetal life and adulthood. The doses of testosterone propionate (TP) used were selected based on previous results showing that doses above 1 mg TP (per rat) increased female fetal testosterone by 80%, increased female anogential distance (AGD) and rescued the seminal vesicles without a high incidence of toxicological effects (Wolf *et al*, 2002).

Testosterone propionate (T5):	5 mgkg ⁻¹ TP in 0.4 ml corn oil, e14.4-21.5 (n=6 litters) subcutaneous injection at 0.4 mlkg ⁻¹ maternal bodyweight
Testosterone propionate (T20):	20 mgkg ⁻¹ TP in 0.4 ml corn oil, subcutaneous (s.c) injection at 0.4 mlkg ⁻¹ maternal bodyweight e14.4-21.5 (n=10) e14.4-15.5 (n=2) e14.4-17.5 (n=2)
Flutamide:	100mgkg ⁻¹ in 1ml corn oil/2.5% DMSO, e15.5-21.5 (n=10) gavage at 1 mlkg ⁻¹ dam bodyweight
Control:	1ml corn oil/2.5% DMSO, e15.5-21.5 by gavage 0.4 mlkg ⁻¹ corn oil, e14.5-21.5 by s.c injection (n=4)

Throughout this chapter, offspring from dams treated while pregnant will be referred to, for example, as “testosterone-exposed” or treated “*in utero*”. This does not mean that fetuses were exposed directly but rather were exposed indirectly via their mother.

Preliminary studies and previous literature (Wolf *et al*, 2002; Wolf *et al*, 2004) suggested that exposure to testosterone resulted in problems with labour and a high incidence of fetal mortality. Therefore, if dams were to give birth to pups for postnatal examination, control

pregnancies were mated on the same day to provide a foster mum for the testosterone-exposed pups if the dam could not litter normally and required the fetuses to be removed by caesarean section. In such instances the control pups were removed from the dam within hours of birth and replaced with the testosterone-exposed pups. No obvious differences were seen in the reproductive tracts of offspring reared by their biological mother compared to those reared by a foster mother.

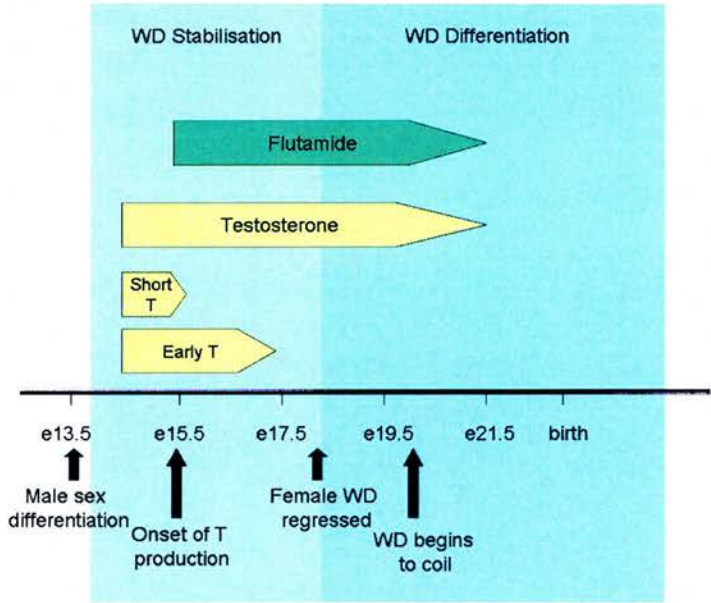


Figure 7.1 Summary of maternal treatment regimes

7.2.2 Tissue collection

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation and fetuses were recovered at e18.5 and e21.5 and killed by decapitation. Postnatal animals, older than pnd10, were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Fetal and postnatal animals were weighed and AGD was measured using digital callipers. Reproductive tracts were recovered by microdissection and photographed using a Leica ICA camera (section 2.3.2.1). Reproductive tracts were analysed microscopically, at the time of dissection, for any gross morphological abnormalities, and tissue was fixed in Bouin's, as detailed in section 2.4. Fetal testes (e21.5) were weighed by members of Prof. Richard Sharpe's lab group, for which I am grateful.

7.2.3 Luminal length analysis

Differentiation of e18.5 and e21.5 WDs was quantified by digitally measuring the luminal length of the epididymal segment of WDs from control and treated animals, as described in section 2.8.

7.2.4 Immunohistochemistry

Immunohistochemistry was performed on isolated rat reproductive tracts fixed in Bouin's, using standard avidin peroxidase protocols in order to highlight the location of proteins of interest, as detailed in section 2.6.1. Briefly, sections were cut from paraffin blocks and mounted on glass slides. Sections were then dewaxed and rehydrated then pressure cooked in citrate buffer if required (Table 7.1). Non-specific binding was blocked before adding the appropriate primary antibody overnight, as detailed in Table 7.1. A biotin labelled secondary antibody was used that had been raised against a species-specific sequence on the primary antibody. This biotinylated secondary antibody signal was amplified by incubation with avidin-biotin conjugated with horseradish peroxidase and the signal was localised using DAB. Sections were counterstained with haematoxylin and mounted with glass coverslips. Further detailed protocols can be found in section 2.6.1.

Table 7.1 Summary of primary antibodies used for immunohistochemistry

Target Antigen	Source	Dilution	Retrieval	Host Species
Androgen Receptor	Santa Cruz	1:50	Citrate	Rabbit
Smooth Muscle Actin	Sigma	1:10000	None	Mouse
Pan-cytokeratin	Sigma	1:200	Citrate	Mouse
Desmin	DAKO	1:400	Citrate	Mouse

7.2.5 Analysis of apoptosis

Cleaved caspase 3 immunostaining was performed on WDs using standard methods, as detailed in section 2.6.1, to stain for apoptotic cells. Very few cleaved caspase 3 positive cells were detected, therefore a detailed stereological analysis was not appropriate and all positive cells were manually counted in each WD using an Axiolab microscope (Carl Zeiss Ltd, Hertfordshire, UK).

7.3 Results

7.3.1 Effects of testosterone exposure

Due to time restraints and in an attempt to reduce the number of animals used, not all endpoints were examined for all animal models discussed. All endpoints were examined using WDs from litters exposed to high dose testosterone (T20, 20 mgkg⁻¹, e14.5-21.5) as these WDs were most likely to highlight any low incidence phenotype, rather than those exposed to lower dose testosterone or testosterone-exposure within shorter windows.

7.3.1.1 Effects of testosterone on dam weight

Exposure to low (T5 - 5 mgkg⁻¹) or high (T20 - 20 mgkg⁻¹) doses of testosterone resulted in a reduction in maternal weight gain at e18.5 and e21.5, compared to controls and relative to bodyweight at e15.5 (Fig. 7.2): this reduction in dam weight gain was comparable in the high and low dose testosterone groups. Exposure to flutamide (100 mgkg⁻¹) also resulted in a small, but non-significant, reduction in dam weight gain: this reduction was enhanced by combined exposure to flutamide (100 mgkg⁻¹) and testosterone (20 mgkg⁻¹) but this was less than that seen after exposure to testosterone alone suggesting that flutamide offers some degree of protection against the growth restriction effects of testosterone (Fig. 7.2).

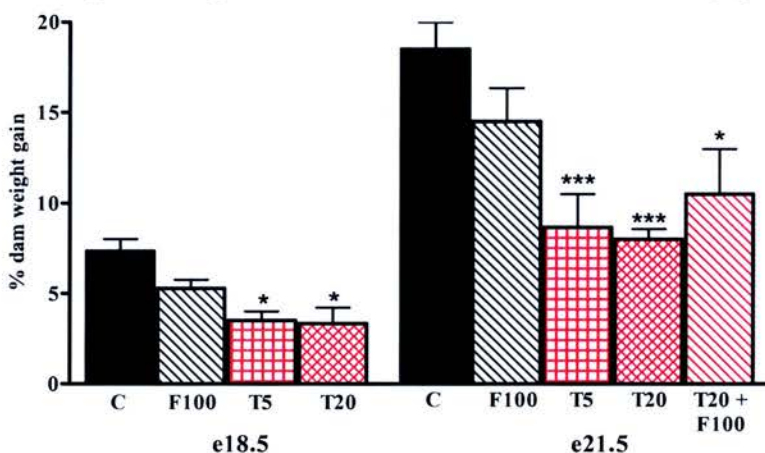


Figure 7.2 Maternal % weight gain at e18.5 and e21.5, relative to bodyweight at e15.5.

Note the reduction in weight gain in dams exposed to low (T5, 5 mgkg⁻¹) or high (T20, 20 mgkg⁻¹) doses of testosterone: this reduction was still seen in dams exposed to testosterone (20 mgkg⁻¹) and flutamide (100 mgkg⁻¹) together. There was also a trend for reduced weight gain in dams exposed to flutamide (100 mgkg⁻¹) during pregnancy, but this was not significant. Values are means \pm S.E.M for 3-8 animals per group. * $p < 0.05$, *** $p < 0.001$, compared to age matched controls.

7.3.1.2 Effects of maternal testosterone exposure on pup weight

At e21.5, there was a significant decrease in fetal bodyweight after exposure to either low (T5, 5 mgkg⁻¹) or high (T20, 20 mgkg⁻¹) doses of testosterone, compared to age matched controls (p<0.01). This reduction in bodyweight was seen in both female and male fetuses from litters exposed to testosterone throughout reproductive development (e14.5-21.5) or during short defined windows early in WD development (e14.5-15.5 or e14.5-17.5) (Fig.7.3). Fetuses exposed to flutamide (100 mgkg⁻¹) and testosterone (20 mgkg⁻¹) in combination also had reduced bodyweight at e21.5, though this reduction tended to be slightly less than that observed in fetuses exposed to testosterone alone (Fig.7.3).

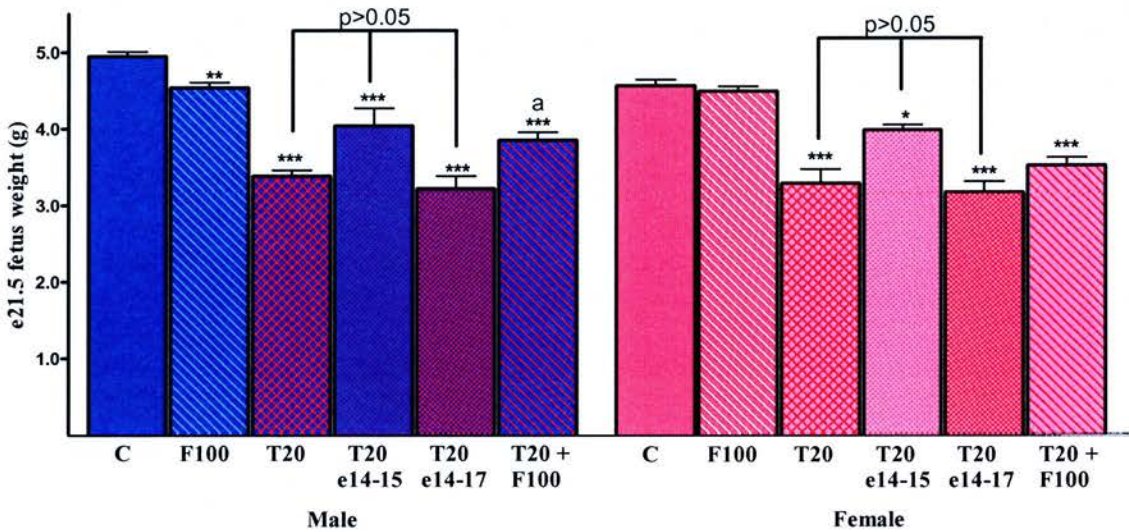


Figure 7.3 Bodyweight at e21.5 in male (blue bars) and female (pink bars) fetuses from control, high dose flutamide (100 mgkg⁻¹), low dose testosterone (T5, 5 mgkg⁻¹) or high dose testosterone (T20, 20 mgkg⁻¹) litters. Note that bodyweight was reduced in male and female fetuses exposed to testosterone either alone or in combination with flutamide (T20 + F100), compared to sex matched controls. Note also that bodyweight was reduced only in male fetuses from litters exposed to flutamide (100 mgkg⁻¹), not in female fetuses. Values are means \pm S.E.M for 9-28 animals per group. p<0.05, *** p<0.001, compared to sex matched controls.

By pnd17, no significant difference was seen in bodyweight of males or females that had been exposed to testosterone (20 mgkg⁻¹) *in utero*, compared to age matched controls (data not shown).

7.3.1.3 Effects of maternal testosterone exposure on fetal reproductive tract

7.3.1.3.1 Anogenital distance

Normally, fetal sex can be determined externally by the distance between external genitalia and the anus (anogenital distance; AGD), with AGD significantly larger in fetal males than in females (Fig. 7.4). Maternal exposure to either dose of testosterone (5 or 20 mgkg⁻¹), had no significant effect on anogenital distance (AGD) in male fetuses at e21.5 but increased female AGD to a length similar to that seen in control male fetuses (Fig. 7.4). This increase was noted in females exposed to testosterone throughout fetal reproductive development (e14.5-21.5) and those exposed to testosterone only during a short early window in development (e14.5-15.5 or e14.5-17.5) (Fig. 7.4). As discussed in chapter 3, exposure to flutamide (100 mgkg⁻¹) reduced male AGD to a length comparable to that of control females; exposure to flutamide (100 mgkg⁻¹) in combination with testosterone (20 mgkg⁻¹; T20 + F100) completely prevented the testosterone-induced increase in AGD in female fetuses and induced a reduction in male AGD, equivalent to that seen after exposure to flutamide alone (Fig. 7.4).

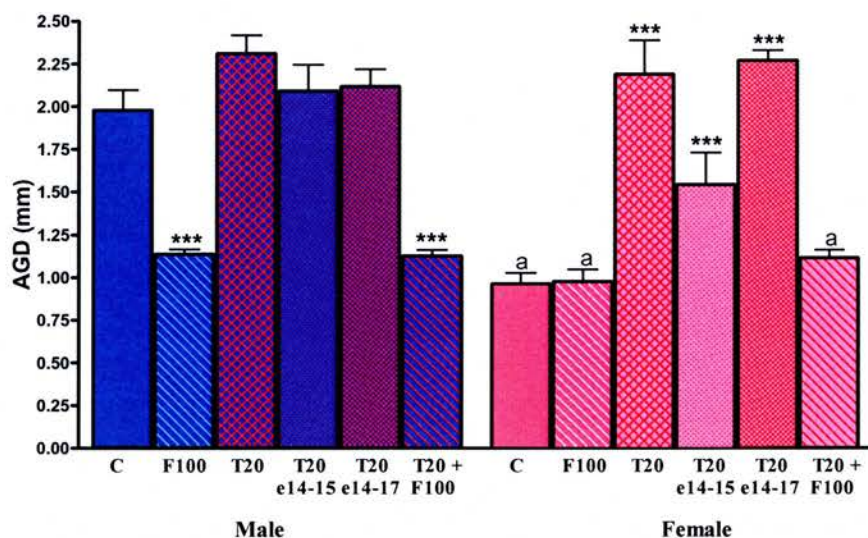


Figure 7.4 Anogenital distance (AGD) in e21.5 male (blue bars) and female (pink bars) fetuses from control, high dose maternal flutamide (100 mgkg⁻¹) and high dose testosterone (20 mgkg⁻¹) litters. Note that AGD was significantly smaller in control female fetuses than in males. AGD was reduced in male fetuses exposed to flutamide alone or in combination with testosterone (T20 + F100) compared to control males. AGD was increased in females exposed to all doses of testosterone to a length comparable to control male AGD. Note also that maternal exposure to flutamide did not alter female AGD ($p > 0.05$), even in combination with testosterone (T20 + F100). Values are means \pm S.E.M for 5-23 fetuses. *** $p < 0.001$ compared to sex matched control AGD, a $p < 0.001$ compared to control male AGD.

AGD continued to be significantly smaller in control females than males at pnd17 ($p < 0.05$): exposure to testosterone (5 or 20 mgkg⁻¹) during gestation permanently increased AGD in both males ($p > 0.05$) and females ($p < 0.001$) resulting in testosterone-exposed female AGD being comparable to that in a control male ($p > 0.05$) (data not shown).

7.3.1.3.2 Prostate development after exposure to testosterone

Analysis of prostate morphology in these studies was guided by Dr Axel Thomson, for which I am grateful. Due to time constraints, this was only analysed in animals exposed to testosterone (5 or 20 mgkg⁻¹) for the entire period of fetal reproductive development (e14.5-21.5).

The prostate begins to bud and develop in male rats at around e18.5: at this age prostatic buds could be identified in females from testosterone-exposed litters (5 or 20 mgkg⁻¹) (data not shown). These prostates were not highly developed and resembled prostates from e18.5 control males. At this age, a pad of inductive mesenchyme can be identified in control females but no epithelial buds can be distinguished (Timms *et al*, 1995; Thomson *et al*, 2002).

In males, the dorsolateral lobe of the prostate develops first with the ventral prostatic lobe budding later in fetal life. By e21.5, budding/branching could be seen in the dorsolateral prostate in some low dose testosterone-exposed females: the extent of this development varied between individuals with some prostates clearly branched while others showed no sign of budding (data not shown). Contrastingly, all females from litters exposed to 20 mgkg⁻¹ testosterone had highly masculinised prostates with budding evident in both the dorsolateral and ventral prostates (Fig. 7.5). These female prostates were not as developed as control male prostates at e21.5 and were estimated to be approximately 1-2 days behind male development.

The prostates from e21.5 male fetuses from both low and high dose testosterone groups were highly developed and masculinised: it is difficult to say objectively whether these prostates were more developed than their age matched controls, but they were at the upper end of the scale of normal development (data not shown).

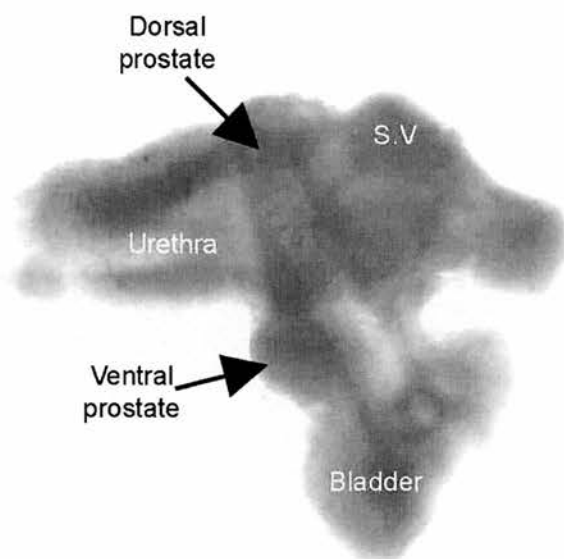


Figure 7.5 Representative urogenital sinus region from an e21.5 female testosterone-exposed (5 mgkg^{-1}) fetus. Note the presence of seminal vesicles (s.v) and both dorsal and ventral prostatic lobes. Note also the presence of epithelial budding in the dorsal prostate.

7.3.1.3.3 Testosterone exposure prevented normal vaginal development

By e21.5, the vagina develops in control females distal to the cervix, alongside the urethra but females exposed to testosterone (5 or 20 mgkg^{-1}) had no obvious vaginal opening (data not shown). There were no obvious abnormalities in the uteri in these animals (data not shown).

7.3.1.3.4 Development of seminal vesicles after testosterone exposure

Seminal vesicle development can be seen in male fetal rats around e18.5 by the presence of a mesenchymal bud. This was noted in females at e18.5 after exposure to both low (5 mgkg^{-1}) and high (20 mgkg^{-1}) dose testosterone but no obvious epithelium was detected, as in age matched males (data not shown). By e21.5, this seminal vesicle mesenchymal bud was still apparent in testosterone-exposed females (5 or 20 mgkg^{-1}) with epithelia evident in some females, particularly from the high dose testosterone litters (Fig. 7.6). No obvious difference was noted in the appearance of the seminal vesicles in testosterone treated males compared to their age matched controls at e18.5 or e21.5 (data not shown).

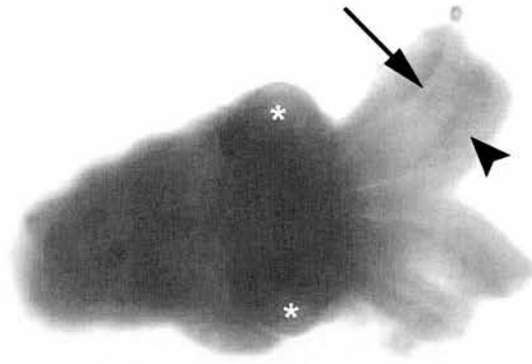


Figure 7.6 Representative urogenital tract from an e21.5 female testosterone-exposed (20 mgkg^{-1}) fetus. Note the presence of seminal vesicles (*) as well as both the MD (arrowhead) and WD (arrow).

7.3.1.3.5 Testosterone exposure alters the position of the ovary

In control females, the ovary is located at the cranial/lateral edge of the kidney at both e18.5 and e21.5. In testosterone-exposed females, the ovary was situated slightly lower at the more caudal pole of the kidney compared to control females; however, the ovary did not descend into the lower abdomen as the testis does by e21.5 in males (data not shown). This apparent descent was not quantified.

7.3.1.3.6 Impact of testosterone exposure on the testis

Exposure to testosterone (20 mgkg^{-1}) resulted in an average 25% reduction in testis weight at e21.5 compared to age matched controls ($p < 0.0001$). This is likely to be due to a reduction in the number of Sertoli cells in these testes (data not shown).

7.3.2 Effects of testosterone exposure on WD development

7.3.2.1 Fetal Wolffian duct

At e18.5 the male WD is a simple straight duct which becomes highly coiled by e21.5 (Fig. 7.7 and 7.8). Exposure to testosterone (5 or 20 mgkg^{-1}) either during the entire window of fetal reproductive development (e14.5-21.5) or during short defined periods early in WD development (e14.5-15.5 or e14.5-17.5) had no obvious impact on male WD development at either e18.5 or e21.5 (Fig. 7.7 and 7.8). As detailed in chapter 3, exposure to flutamide (100 mgkg^{-1}) reduced the coiling normally seen in the male WD by e21.5 (Fig. 7.8) and exposure to testosterone (20 mgkg^{-1}) in combination with flutamide did not prevent this impaired coiling in male WDs at e21.5 (Fig. 7.8).

In normal females, the WD has almost completely regressed by e18.5, with only a small remnant evident at the cauda (Fig.7.7 and section 6.4.1.1). At e18.5 and e21.5, WDs were identified in many females exposed to both high (20 mgkg⁻¹) and low (5 mgkg⁻¹) doses of testosterone (Fig. 7.7 and 7.8): these complete WDs were noted in all e21.5 females exposed to 20 mgkg⁻¹ testosterone (Fig. 7.10) and even showed some degree of coiling, although this was much less than that seen in control male WDs by e21.5 (Fig. 7.8). Variation was seen in the female WD morphology after exposure to 5 mgkg⁻¹ testosterone at both e18.5 and e21.5 (Fig. 7.7 – 7.10): in 25% of animals no WD was seen, in 53% of fetuses the WD could only be identified at the caput while in the remaining 22% a full length WD was noted, with coiling even evident at the caput and cauda (Fig. 7.7 – 7.10). Exposure to testosterone (20 mgkg⁻¹) within short defined windows early in WD development (e14.5-15.5 or e14.5-17.5) also rescued the female WD in some animals and was even able to stimulate coiling by e21.5, particularly at the caput (Fig 7.7 and 7.10). However, exposure to flutamide (100 mgkg⁻¹) in combination with testosterone (20 mgkg⁻¹) prevented this WD rescue and, in this treatment group, no WD was identified in any female examined at e21.5 (Fig. 7.7 and 7.10).

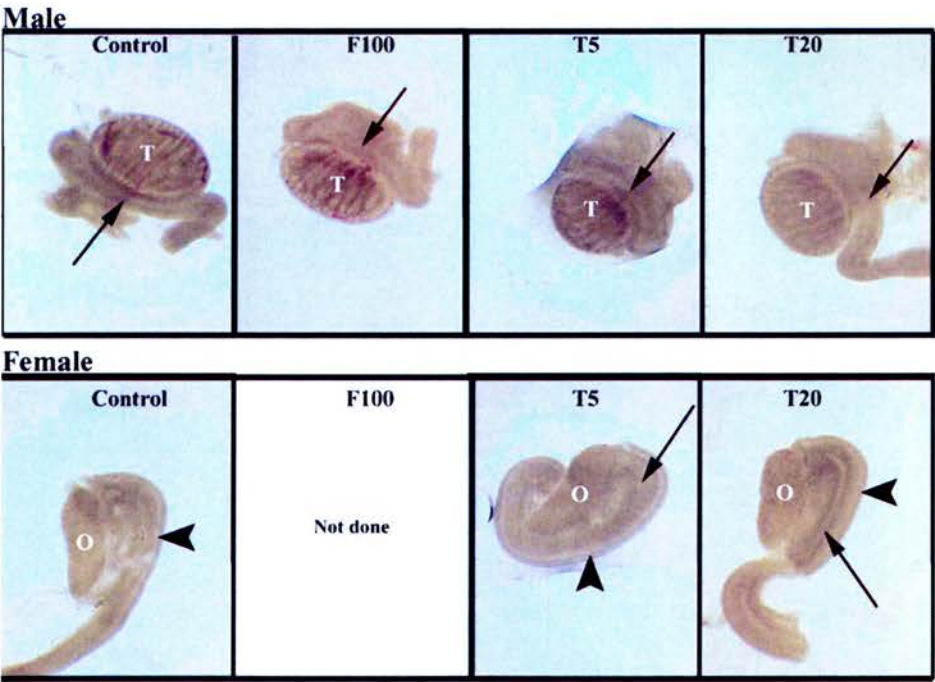


Figure 7.7 Representative WDs from e18.5 male and female control, flutamide-exposed (F100, 100mgkg⁻¹) and testosterone-exposed (T5, 5 mgkg⁻¹ or T20, 20 mgkg⁻¹) fetuses. Note that no obvious difference can be seen in the male WD after exposure to flutamide or testosterone (arrow, top row). Note also that no WD can be seen in the control female at e18.5 whereas in testosterone-exposed females (arrow, T5 or T20) the WD can be clearly identified lying medial to the MD (arrowhead). All images are at the same magnification. T – testis, O – ovary

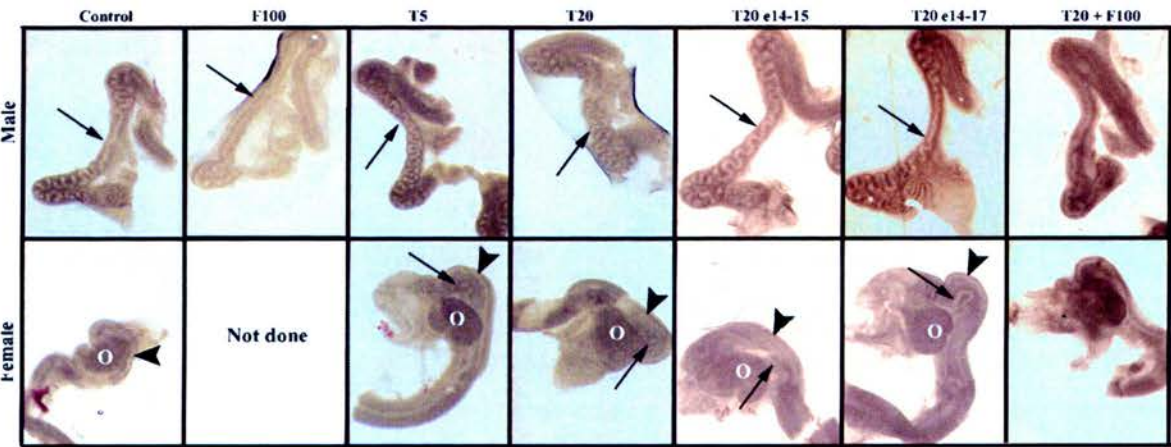


Figure 7.8 Representative WDs from e21.5 male and female control, flutamide-exposed (F100, 100mgkg⁻¹) and testosterone-exposed (T5, 5 mgkg⁻¹ or T20, 20 mgkg⁻¹) fetuses. Note that the male WD (arrow) was highly coiled at e21.5 and this was not affected by exposure to either dose of testosterone within any time window. Flutamide exposure inhibited this coiling in male WDs at e21.5 and testosterone was not able to rescue this effect (T20 + F100). Note also that at e21.5, no WD was evident in the female but a full WD (arrow) was evident medial to the MD (arrowhead) in females after exposure to testosterone. Exposure to flutamide in combination with testosterone (T20 + F100) prevented this rescue of the female WD. All images are at the same magnification. O- ovary

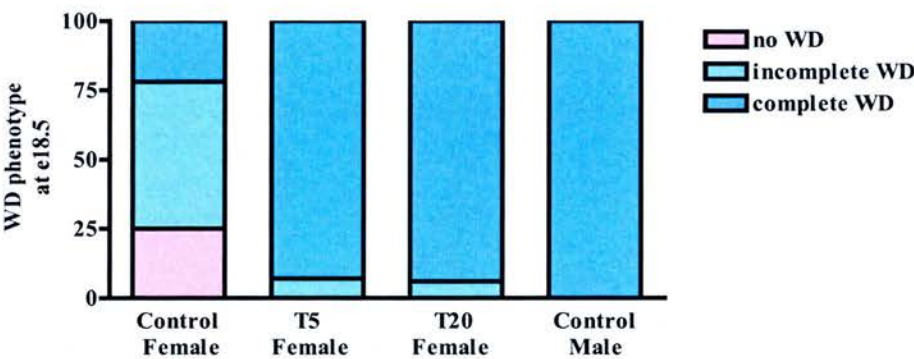


Figure 7.9 Prevalence and morphology of WDs at e18.5 in male and female control and testosterone-exposed (T5, 5 mgkg⁻¹ or T20, 20 mgkg⁻¹) fetuses. Note that a complete WD was identified in all control males whereas in 25% of control female fetuses no WD was identified, a complete WD was noted in 22% and in 53% an incomplete WD was seen. After exposure to testosterone (T5 or T20), the majority of females had a complete WD. Values are means for 3 litters per group.

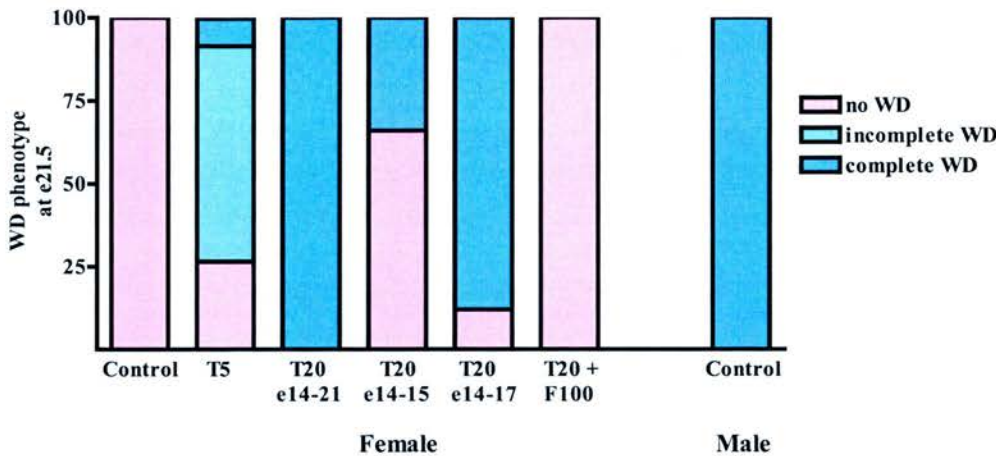


Figure 7.10 Prevalence and morphology of WDs at e21.5 in male and female control and testosterone-exposed (T5, 5 mgkg⁻¹ or T20, 20 mgkg⁻¹) fetuses. Note that a complete WD was seen in all control males but no WD was seen in any control female examined. Exposure to 20 mgkg⁻¹ testosterone between e14.5-21.5 rescued the WD in all females whereas exposure to 5 mgkg⁻¹ only partially rescued the WD in some females. Note however that WDs were only identified in some females after exposure to testosterone (20 mgkg⁻¹) between e14.5-15.5 or between e14.5-17.5. Note that exposure to flutamide (100 mgkg⁻¹) in combination with testosterone (T20 + F100) prevented the testosterone-induced rescue of the female WD. Values are means for 3 litters per group.

These qualitative observations regarding the impact of testosterone exposure on the male WD were quantified by measuring the length of the WD lumen. At e18.5, no significant difference was noted in the luminal length of the male WD after exposure to flutamide (100 mgkg⁻¹) or testosterone (T5, 5 mgkg⁻¹ or T20, 20 mgkg⁻¹), compared to age matched controls (Fig.7.11). Furthermore, there was no significant difference in the luminal length of the testosterone-exposed (20 mgkg⁻¹) female WD compared to the male control WD (Fig.7.11). Quantification of luminal length confirmed the reduction in WD coiling at e21.5 in males exposed to flutamide (100 mgkg⁻¹) alone or in combination with testosterone (T20 + F100, 20 mgkg⁻¹), compared to age matched controls (Fig. 7.12). Exposure to 5 or 20 mgkg⁻¹ testosterone had no significant impact on male WD luminal length at e21.5 (Fig. 7.12). It is worth noting that the luminal length of the female testosterone-exposed (20 mgkg⁻¹) WD was significantly reduced compared to both the control and testosterone-exposed male WD (Fig.7.12).

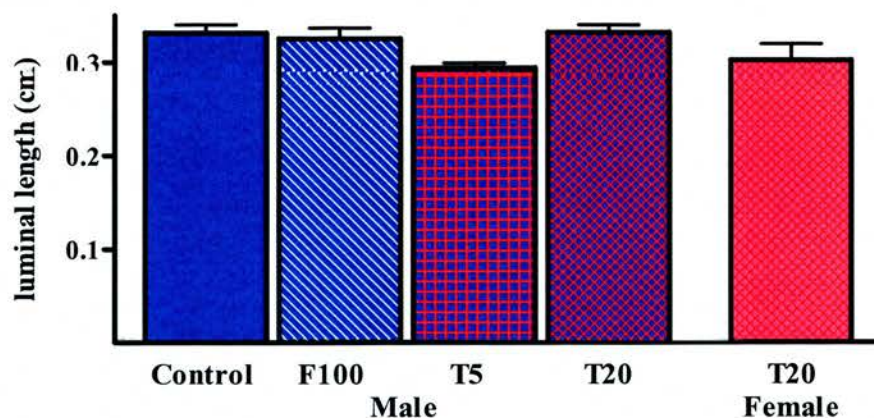


Figure 7.11 Quantification of coiling (= luminal length) of e18.5 WDs from control, flutamide-exposed (100 mgkg^{-1}) and testosterone-exposed ($T5$, 5 mgkg^{-1} or $T20$, 20 mgkg^{-1}) male (blue bars) and female (pink bar) fetuses. Note that no significant difference was seen in male or female WD luminal length after exposure to flutamide or testosterone, compared to luminal length of male control WDs (solid blue bar). Values are mean \pm S.E.M for 10-21 WDs.

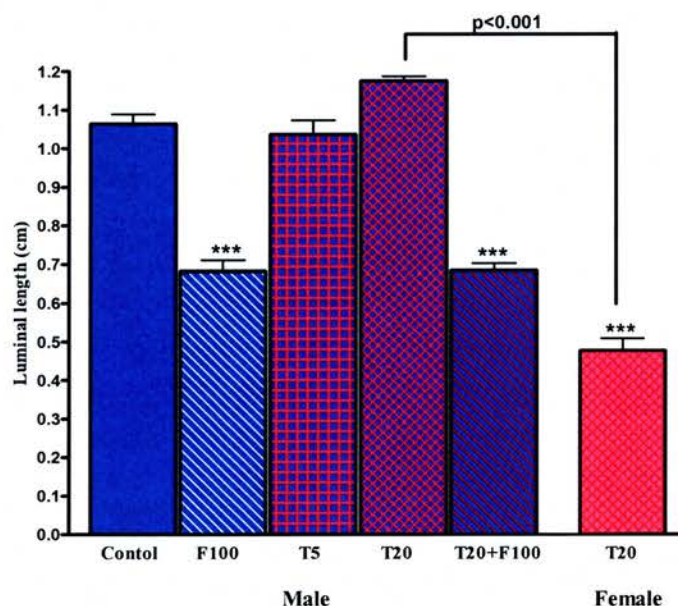


Figure 7.12 Quantification of coiling (= luminal length) of e21.5 WDs from control, flutamide-exposed (100 mgkg^{-1}) and testosterone-exposed ($T5$, 5 mgkg^{-1} or $T20$, 20 mgkg^{-1}) male (blue bars) and female (pink bar) fetuses. Note that exposure to flutamide alone (F100) or in combination with testosterone ($T20 + F100$) significantly reduced male WD luminal length compared to control males. Exposure to 5 or 20 mgkg^{-1} testosterone ($T5$ or $T20$) had no impact on male WD luminal length. Note also that testosterone-exposed female WD luminal length was significantly shorter than both control and testosterone-exposed (20 mgkg^{-1}) male WDs. Values are mean \pm S.E.M for 11-30 WDs. * $p < 0.05$, *** $p < 0.001$, compared to male control WD luminal length.

7.3.2.2 Postnatal WD-derived tissues

The epididymis and vas deferens did not appear obviously different in the postnatal male (pnd17, 42 or 70) after exposure to testosterone (5 or 20 mgkg⁻¹) during gestation, compared to age matched controls (data not shown).

Epididymides/vasa deferentia could still be clearly identified in many of the testosterone-exposed (5 or 20 mgkg⁻¹) postnatal females (pnd17, 42 and 70). Some degree of coiling could be seen in many epididymides but they were considerably underdeveloped compared to age matched control male epididymides and were incomplete in several females. (data not shown).

Upon histological analysis, it was clear that these testosterone-rescued postnatal female WDs were organised into the same compartments as those seen in the normal male WD (discussed in chapter 4). Cross sections of epithelium were seen, highlighting the coiling seen in regions of the female rescued WD: these were surrounded by rings of smooth muscle while the stroma more distant to the epithelium was negative for smooth muscle actin (SMA) (Fig. 7.13).

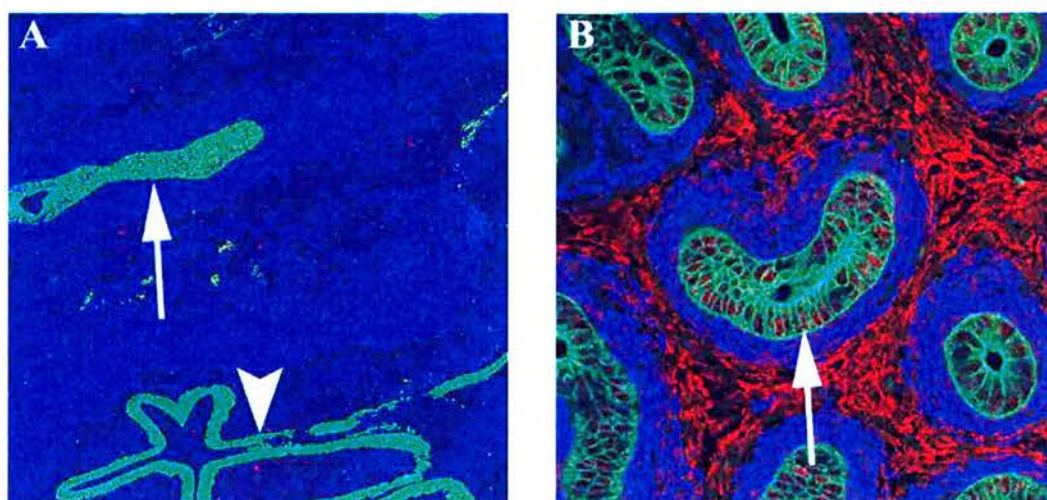


Figure 7.13 Representative WDs from pnd17 females exposed to exogenous maternal testosterone in utero (20 mgkg⁻¹) immunostained for smooth muscle actin (SMA, blue), cytokeratin (green) and androgen receptor (AR, red) to highlight the different cellular compartments. Note in A that the WD (arrow) can be seen lying alongside the developing uterus (arrowhead). Several cross sections of epithelium (immunostained green) can be identified in panel B, highlighting the coiling seen in the caput of the female rescued WD. Note also the smooth muscle ring in the stroma immediately adjacent to the epithelium in the female testosterone-rescued WD.

7.3.3 Histology of Wolffian ducts exposed to exogenous maternal testosterone

7.3.3.1 Prevalence of apoptosis in testosterone-exposed WDs

As in control male WDs, apoptotic cells were rarely detected in either the epithelial or stromal compartments of WDs from male or female testosterone-exposed (20 mg kg^{-1}) fetuses at e21.5 (data not shown).

7.3.3.2 AR expression in testosterone-exposed WDs

The androgen receptor (AR) protein was immunolocalised to both stromal and epithelial cell nuclei in the male WD at e21.5: this expression pattern was not obviously altered by testosterone-exposure (Fig. 7.12). In the control female, AR was also immunolocalised to the stroma and epithelium at e18.5 (section 6.4.1.3). It is not surprising therefore that AR was expressed in the stroma and epithelium of the female testosterone-exposed WD at e21.5 but not in the MD (Fig. 7.14).

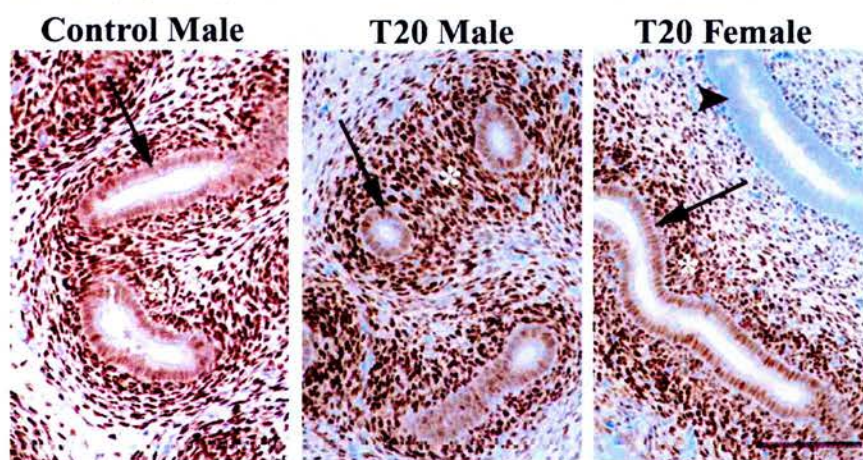


Figure 7.14 *Immunoexpression of androgen receptor (AR) in the WD of male and female control and testosterone-exposed (T20, 20 mg kg^{-1}) fetuses at e21.5. Note the AR expression (brown) in the epithelium (arrow) and stroma (*) in control male and testosterone-exposed male and female WDs while the female MD (arrowhead) is immuno-negative for AR. Scale bar = $100 \mu\text{m}$*

7.3.3.3 Presence of epithelium in testosterone-exposed female WDs

Immunohistochemistry for cytokeratin, an epithelial cell marker, highlighted the presence of an apparently normal epithelium in the testosterone-exposed female WD at e21.5 (Fig. 7.15): the presence of several epithelial cross sections in regions of these WDs was indicative of the coiling seen during gross analysis (as discussed in section 7.3.1.4.2).

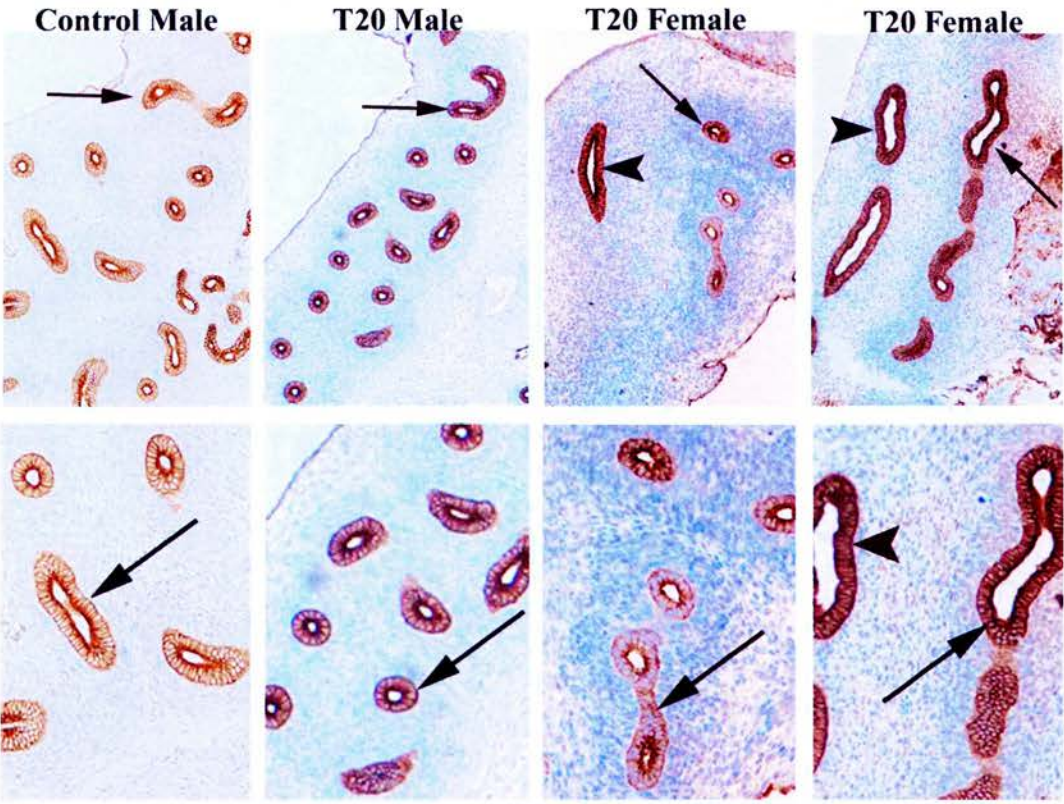


Figure 7.15 Representative e21.5 WDs from control and testosterone-exposed (T20, 20 mgkg^{-1}) males and females immunostained for cytokeratin (brown). Note the presence of epithelium in the testosterone-exposed female WD (arrow), lying medial to the MD (arrowhead). This epithelium seemed comparable to that seen in both control and testosterone-exposed male WDs (arrow).

7.3.3.4 SMA expression in testosterone-exposed WDs

Immunoexpression of smooth muscle actin (SMA), a marker of smooth muscle differentiation, was evident in the stromal cells immediately adjacent to the epithelium in control e21.5 male WDs: this expression was not altered in the male WD by exposure to testosterone (20 mgkg^{-1}) (Fig. 7.16). SMA was similarly expressed in the stroma of female testosterone-exposed WDs at e21.5 (Fig.7.16).

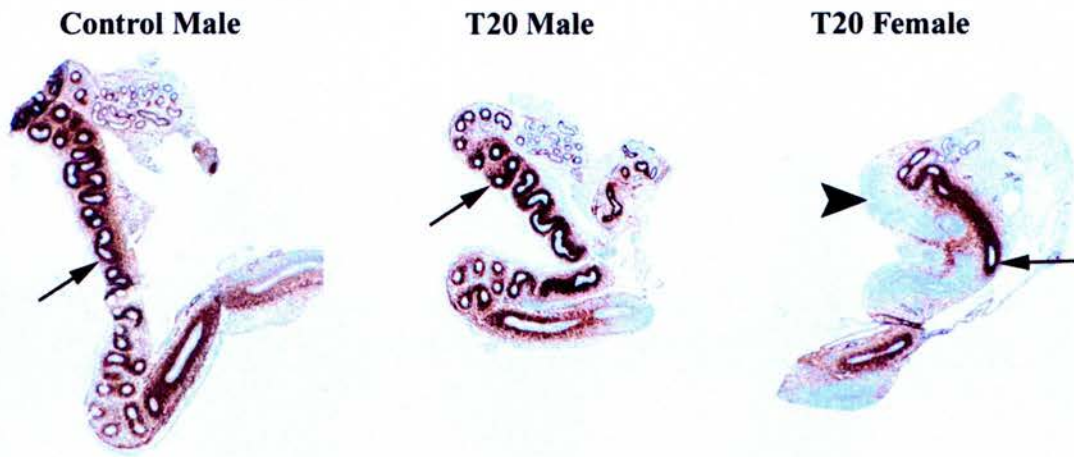


Figure 7.16 *Immunoexpression of smooth muscle actin (SMA) in the stroma in control male and testosterone-exposed (T20, 20 mgkg⁻¹) male and female WDs. Note that SMA is strongly expressed in the stroma immediately adjacent to the epithelium in control and testosterone-exposed male WDs as well as in female testosterone-exposed WDs (arrow). Note however that the stroma surrounding the MD (arrowhead) is negative for SMA. All images are at the same magnification.*

7.3.3.5 Desmin expression

Desmin was expressed in the stromal compartment of the control male WD at e15.5-21.e (as discussed in section 4.4.3.2) and was detected by immunohistochemistry in the stroma of both male and female testosterone-exposed (20 mgkg⁻¹) WDs at e21.5 (Fig. 7.17).

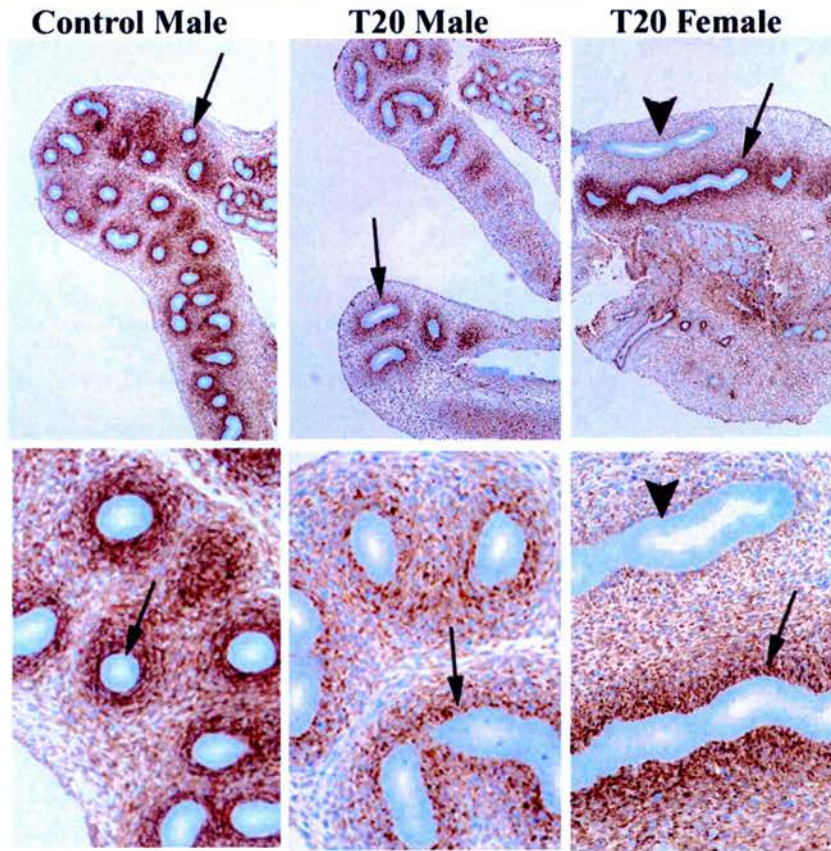


Figure 7.17 Desmin expression in control male and testosterone-exposed (20 mg kg^{-1}) male and female WDs at e21.5. Note that desmin is expressed in the WD (arrow) stroma and is not obviously altered by exposure to testosterone. Note also that desmin can be weakly immunolocalised to the stroma surrounding the MD (arrowhead).

7.4 Discussion

As discussed in chapter 6, the female rat WD regresses between e16.5-18.5 and this is believed to be due to the lack of androgens. However, studies in Tfm mice and rats exposed to DBP and flutamide in combination raised the question of whether androgens alone were acting to stabilise the male WD and whether some other compensatory factor may play a role in rescuing the male WD. Studies were therefore undertaken to investigate whether exposure to exogenous androgens during gestation was sufficient to rescue the female WD or whether unknown testicular factors may also be required in male WD development.

Previous studies have shown that exposing pregnant rats to various doses of testosterone masculinises the female offspring to varying degrees (Jost *et al*, 1973; Stinnakre, 1975; Wolf *et al*, 2000; Wolf *et al*, 2002; Padmanabhan *et al*, 2006). However, little is known about the ability of exogenous testosterone to rescue the female WD. Pregnant rats were exposed to testosterone propionate (5 or 20 mgkg⁻¹) throughout the period of fetal reproductive development (e14.5-21.5) and various endpoints were examined. The only obvious effect noted in the male fetal reproductive tract was that fetal testes were significantly smaller at e21.5, compared to age matched controls. This is in agreement with previous studies (Wolf *et al*, 2002). Development and differentiation of the male WD was not dramatically affected by exposure to exogenous testosterone, with coiling not initiated any earlier, suggesting therefore that the initiation of WD development is not dependent by the level of testosterone available. Contrastingly, female fetal reproductive development was dramatically altered by maternal exposure to exogenous testosterone. In testosterone-exposed female fetuses AGD was increased to a length similar to control males, prostatic buds developed where the vagina would normally develop, seminal vesicles were apparent, the WD was rescued, and the ovary appeared slightly more descended. These findings agree with previous studies highlighting that exogenous testosterone alone is capable of masculinising the female fetus (Greene, 1939; Swanson and Werff ten Bosch, 1965; Ogawa and Nozawa, 1969; Stinnakre, 1975; Wolf *et al*, 2002; Manikkam *et al*, 2004; Wolf *et al*, 2004; Padmanabhan *et al*, 2006). Furthermore, the WD was not only rescued in females exposed to maternal testosterone (5 or 20 mgkg⁻¹) but some degree of differentiation and compartmentalisation occurred, as seen by the initiation of coiling at the epididymal caput in particular by e21.5 while the prospective vas deferens remained straight. By this age all normal female WDs had completely regressed (discussed in chapter 6), therefore confirming that exposure to testosterone alone, between e14.5-21.5, is sufficient to stabilise and even partially differentiate the female WD. It is worth noting however that coiling was never as dramatic in the testosterone-exposed

female WD as that seen in control male WDs by e21.5. There are various explanations for this. Firstly, it is possible that the doses of testosterone given in this study were not sufficient to stimulate coiling fully and that dosing with an even higher level of testosterone propionate, or even with the more potent androgen DHT, may result in more dramatic coiling in the female rescued WD similar to that seen in the normal male. This hypothesis is supported by results reported in chapter 3, highlighting that exposure to 100 mgkg⁻¹ flutamide did not prevent stabilisation of the male WD but was able to inhibit its subsequent coiling, thus suggesting that higher levels of testosterone were required for differentiation than that needed for WD stabilisation in the male. Bentvelsen and colleagues (1995) exposed pregnant rats to DHT and as expected this was able to rescue the female WD, however, they do not mention the degree of coiling initiated in these studies. It is difficult to compare the levels of testosterone used in many previous studies as they often express testosterone doses per rat rather than by dam weight but it is fair to claim that the doses of testosterone used in this thesis are not significantly different from previous studies. It is worth noting however that Greene and colleagues (1939) reported that higher doses of testosterone did elicit a greater degree of epididymal coiling but images are not provided to allow comparisons with the coiling reported in this thesis.

Secondly, the female rescued WD may be physically prevented from fully coiling due to the presence of the normal MD situated lateral to the WD in females. Alternatively, it may be that testosterone needs to be directly delivered down the lumen of the WD, rather than via systemic exposure. Male WDs are exposed to high levels of testosterone delivered directly to the epithelium down the lumen from the testis, possibly resulting in the epithelium being exposed to greater levels of testosterone than the more distal stroma. It is arguable that, in testosterone-exposed female WDs, the stroma will be exposed to similar or even higher levels of testosterone than the epithelium is, which may be sufficient to permit WD stabilisation but has an impact on subsequent differentiation. This hypothesis is substantiated by studies in Wnt4 knockout mice (Heikkila *et al*, 2005). These female mice are born with complete WDs showing a considerable degree of coiling, although this coiling is still not as dramatic as that seen in a newborn male WD (Heikkila *et al*, 2005). This rescue and coiling of the WD is believed to be due to the production of testosterone by the fetal ovary: this testosterone is likely to be delivered down the lumen of the female WD and thus may explain why Wnt4 KO females have a greater degree of coiling than that seen in these studies after exogenous maternal testosterone exposure. However, it is worth noting that these females have no MD (Heikkila *et al*, 2005) as Wnt4 is required for its formation thus it cannot be ruled out that the MD physically prevents the testosterone-exposed female

WD from fully coiling. However, it should be pointed out that these studies are in mice, rather than rats. No increase was noted in the level of plasma DHT in these KO females compared to their wild-type littermates and levels remained considerably lower than that detected in male mice (Heikkila *et al*, 2005). Furthermore, AGD and the external genitalia were not masculinised in Wnt4 KO females suggesting that the levels of testosterone produced by the ovary were insufficient to masculinise the more peripheral tissues, which are dependent on the more potent DHT in males.

Studies reported in chapter 5 highlighted that stabilisation and patterning of the male WD were established by androgens between e15.5-17.5 and high levels of androgen action were less important later during differentiation of the WD. Exposing fetuses to exogenous maternal testosterone further reinforces the hypothesis that this early time point is a critical window of androgen action even in the female WD as exposure to testosterone (20 mgkg⁻¹) only between e14.5-15.5 or between e14.5-17.5 was able to rescue the WD in many female fetuses and even resulted in the initiation of coiling by e21.5. This is in agreement with studies previously undertaken showing that injecting rats with 30mg testosterone propionate between e15-17 resulted in females with well developed WDs while exposure between e8-10 had no effect on the female reproductive tract (Ogawa and Nozawa, 1969). It should be noted, however, that testosterone propionate (TP) has long lasting effects and is reported to increase circulating testosterone levels for 12-24 h in male rats after a single intramuscular injection of 1 mg (Keating and Tcholakian, 1983). It is possible that testosterone propionate may still be present in the mother's body a few days after the final exposure, so the window of testosterone exposure cannot be tightly defined in these studies: it is assumed though, that if the final exposure of testosterone was on e15.5, the majority of testosterone will be cleared from the dams by e18.5. Furthermore, since TP is aromatisable it is possible that some of the testosterone injected into the pregnant dam will be converted into oestradiol in the placenta (Padmanabhan *et al*, 2006), therefore the exact dose of testosterone that the fetus was exposed to cannot be defined. Studies carried out by Wolf and colleagues showed that injecting 1 mg testosterone propionate into a pregnant rat results in testosterone levels increasing from 0.04 ng to 0.09 ng per female fetus while little difference was noted in male testosterone levels (0.25-0.27 ng) (Wolf *et al*, 2004).

Examination of postnatal females that had been exposed to exogenous maternal testosterone (5 or 20 mgkg⁻¹) during gestation highlighted that the rescued female WD often persisted into postnatal life, even though exposure to maternal testosterone ceased at birth. This suggests that patterning of the fetal WD is established early in reproductive development (e14.5-17.5) and, once stabilised, the female WD persists postnatally. This is in contrast to

males exposed to flutamide *in utero*, in which WD-derivatives were present prior to puberty but were often absent by adulthood (discussed in chapter 3). This contrast may highlight fundamental differences between the male and female WD and the way in which they respond to androgens during puberty or may simply be due to the low number of adult testosterone-exposed females examined in this study and requires further investigation.

Histological analysis of the testosterone-rescued female WD revealed no obvious differences in the histological organisation, compared to control male WDs. AR was expressed in both the stroma and the epithelium in the fetal female WD, as seen in the male WD highlighting its capability to respond to androgens. This is in agreement with previous studies showing that exposure to DHT from e11-20 rescued the female WD in female fetuses and resulted in AR expression pattern similar to that of a male (Bentvelsen *et al*, 1995). The stromal and epithelial compartments could easily be identified in the rescued female WD by the same markers as in the control male WD (as discussed in chapter 4) with simple columnar epithelial cells lining the lumen of the duct directly surrounded by a smooth muscle actin (SMA) positive inner stromal compartment. Desmin, an early muscle differentiation marker, was also detected in the stroma of the female WD, highlighting the normal differentiation of the mesenchyme into muscle as seen in the male WD. These results highlight the normal, but underdeveloped, morphological differentiation of the female rescued WD compared to control male WDs; however it cannot be ascertained if the rescued female WD has functionally differentiated.

In order to investigate the relative levels of testosterone exposure induced exogenously in comparison to that required for normal male fetal development, pregnant dams were exposed to both flutamide (100 mgkg⁻¹) and testosterone (20 mgkg⁻¹) in combination. Male offspring had WDs similar to those seen after exposure to flutamide alone (see chapter 3) while females had no WD by e21.5. Furthermore, female AGD was unaffected by testosterone exposure but male AGD was reduced to a length similar to control females. These results suggest that 20 mgkg⁻¹ testosterone was unable to prevent the effects of flutamide, highlighting that even the high dose of testosterone used in these studies is relatively low compared to endogenous male levels and yet it is still capable of masculinising female fetuses. These results are comparable with studies examining the effects of combined exposure to testosterone and vinclozolin in which it was shown that vinclozolin was able to prevent testosterone-induced masculinisation of the female fetus (Wolf *et al*, 2004). Furthermore, if Wnt4 KO mice are exposed to 100 mgkg⁻¹ flutamide *in utero*, no WD is seen in the newborn female thus confirming that flutamide can antagonise the effects of testosterone on the female WD (Heikkila *et al*, 2005).

Exposure to testosterone alone resulted in reduced maternal weight gain and subsequent reduced fetal bodyweight of both males and females at e21.5. This was noted in both low (5 mgkg⁻¹) and high (20 mgkg⁻¹) dose testosterone exposure groups as well as in fetuses only exposed to testosterone within short defined periods (e14.5-15.5 and e14.5-17.5). The effects of testosterone on fetal bodyweight are thought to be regulated through the mother, rather than directly affecting the fetus as is believed to be the mechanism behind the testosterone-induced alterations to the reproductive tract. It is known that inappropriate steroid hormone signalling during fetal life can affect neonatal growth and testosterone-induced intrauterine growth retardation (IUGR) has been reported previously (Wolf *et al*, 2002; Wolf *et al*, 2004; Steckler *et al*, 2005; Carlsen *et al*, 2006; Padmanabhan *et al*, 2006). Examples of this hormone induced IUGR can also be seen in humans as patients with polycystic ovarian syndrome (PCOS) have an increased prevalence of babies born small for gestational age (Sir-Petermann *et al*, 2005). The effects of testosterone on fetal bodyweight were not prevented by combined exposure to flutamide, unlike in the fetal reproductive system. This is again consistent with studies showing that exposure to vinclozolin could not prevent the testosterone induced decrease in fetal bodyweight (Wolf *et al*, 2004). It is not immediately clear why flutamide or vinclozolin can antagonise the testosterone effects in the reproductive tract but not the IUGR, but it may be due to the former being a direct effect of testosterone on the fetus while the latter is a maternal effect. It is unknown, however whether this is the result of testosterone action directly in the mother or the result of this testosterone being converted into oestradiol since oestrogens are also known to cause a decrease in maternal weight gain and fetal birth weight (Padmanabhan *et al*, 2006).

7.5 Conclusion

Exposure to maternal testosterone during late gestation did not obviously affect male reproductive development however the female reproductive tract was masculinised. These studies confirm that testosterone alone is capable of rescuing the fetal WD and any other testicular factors are not essential for WD stabilisation if sufficient testosterone is available. However, the rescued female WD never differentiated as fully as the male WD, possibly due to insufficient levels of testosterone or differences in testosterone delivery between normal males and testosterone-exposed females. As in normal males, testosterone is vital in the female during the early phase of WD development (e14.5-17.5) in order to rescue the WD and is even able to initiate some degree of coiling. Exposure to flutamide concomitantly with testosterone was able to prevent the effects of testosterone on the female reproductive tract.

8 Final Discussion

Adult WD-derived organs are vital for the maturation and transport of sperm, therefore normal development of these structures is fundamental for normal male fertility. It has been previously shown by many researchers that impaired androgen action in the fetus interferes with this developmental process. However, most researchers have examined the effects in adult tissues, rather than in the fetus, and as yet the mechanisms involved are not understood. The main objectives of this thesis were therefore: (1) to examine the role for androgen action in fetal WD development, (2) to investigate the cellular and molecular mechanisms involved in fetal WD development and their perturbation by anti-androgen treatment, (3) to investigate the role for stromal-epithelial interactions in WD development.

8.1 The role for androgens in WD development

There have been several previous studies investigating the effects of interfering with androgen action in the fetal reproductive tract using either AR knockout mice or exposure to anti-androgenic compounds *in utero* (section 1.5.2.2). These studies reported that the majority of these adult males lacked any WD-derived structures, but it was unknown whether this was due to failure of WD stabilisation or due to 'post-differentiation' degeneration. One of the main findings of this thesis is that the flutamide regime used in these studies did not prevent stabilisation of the WD but impaired its subsequent convolution and differentiation into its adult derivatives. This highlighted WD development as a bi-phasic process with WD stabilisation apparently requiring lower levels of androgen action or even acting by an alternative mechanism than that regulating WD differentiation. It was puzzling why WD stabilisation was not affected by administration of a dose of flutamide, which is approximately 4 fold higher than the dose required to prevent prostate formation and induce feminisation of the external genitalia. Insight from models in which androgen action is more completely blocked has furthered our understanding of this. The models used in this thesis to study this were three-fold: natural WD regression in female rats, WD regression in male Tfm mice, and WD development in male rats exposed to DBP + flutamide (chapter 6).

Studies in the female provide a model in which to study the effects on the WD of a lack of androgen action and this provided a unique opportunity to compare the impact on the WD of artificially reducing androgen action in males (Chapter 6). In our rat colony, the female WD regresses between e16.5-e18.5: there are similarities and differences in the cellular mechanisms involved in the natural regression of the female WD compared to the abnormalities induced in the male WD by flutamide exposure (Chapter 6). Few previous

studies have undertaken detailed examination of the timing of, and mechanisms involved in, WD regression in females, therefore these studies offer new insight. It is worth highlighting that this model is limited in the study of WD regression/development as it does not take into account any fundamental differences there may be between the male and female WDs. It is presumed that male and female WDs are identical and that masculinisation is solely dependent on the presence of androgens but this has not been experimentally proven. The testicular feminised (Tfm) mouse, the mouse equivalent of human CAIS in which the AR is functionally inactive, offers an opportunity to investigate the impact of the complete absence of androgen signalling on the male WD. Furthermore, in order to investigate the impact of severely reduced androgen action on the male WD, rats were exposed to flutamide (100 mgkg⁻¹) and DBP (500 mgkg⁻¹) simultaneously. It was expected that in the complete or near complete absence of androgen action in these animals, the male WD would degenerate at a similar time and by a similar mechanism to that observed in the regressing female WD. However, this was not the case. In both model systems, the male WD was still apparent and looked relatively normal with a patent lumen at an age when the WD had completely degenerated in the female littermates. By birth however, the male WD had degenerated and only rudimentary tissue was observed in both Tfm mice and DBP + flutamide-exposed rats. These studies further highlight the essential role for high levels of androgen action in WD development, however since the early WD in androgen-deprived males does not regress at the same age as in the female, this raises the question of whether another factor may be trying to compensate for the severe reduction in, or absence of, androgen action. This may also explain why the flutamide-exposed WD does not obviously differ from age-matched control WDs at e17.5-19.5. It has always been presumed that androgens are the sole factor mediating male reproductive tract development, therefore this phenomenon warrants further investigation in order to characterise better factors that may contribute to this compensatory role. Further studies in both Tfm mice and DBP + flutamide-exposed rats, examining WD histology and gene expression may provide further insight into the mechanisms involved in WD development.

A possible factor that may compensate for the lack of androgen action in Tfm mice and DBP + flutamide-exposed rats is InsI3. Like testosterone, InsI3 is secreted by the fetal Leydig cells and therefore has the potential to be transported directly into the WD in a similar manner to testosterone (Adham *et al*, 2000; McKinnell *et al*, 2005). InsI3 and its receptor, LGR8, are expressed in the developing WD therefore there is a potential role for this signalling pathway in WD development (unpublished data and personal communication with Bernard Jegou). InsI3^{-/-} mice do not show any obvious signs of impaired WD development,

however testosterone production is normal in these animals and could be sufficient to mediate normal WD development (Adham *et al*, 2000). It would be interesting to examine the effect on the developing WD of ablating both androgen and *Ins13* action. This could be investigated by generating a double knockout mouse from *Tfm* and *Ins13*^{-/-} and examining whether the male WD regresses at the same time as the female WD in these animals. These mice have already been generated by Adham and colleagues (2000) but there are no reports on the timing of WD regression in these male mice. Communication has been established with Adham and colleagues to source these mice in order to undertake this investigation.

Another potential factor which may play a compensatory role in WD development in the absence of androgen action is oestrogen. In the reproductive tract, testosterone has the potential to be aromatised into oestradiol. Oestradiol could have a direct effect on the WD, since the WD expresses ER β , or alternatively oestrogens could act indirectly via the efferent ducts which express ER α . In *Tfm* males, testosterone production is increased but there is no functional AR for it to bind to, but this testosterone could be aromatised into oestradiol which might contribute to why the male *Tfm* WD regresses later than does the normal female WD. Lack of oestrogen action in the aromatase KO (ArKO) mice has no obvious impact on WD development (Fisher *et al*, 1998) but as in the *Ins13*^{-/-} mice, testosterone action is unaffected in these animals therefore it would be interesting to examine the effects of flutamide exposure in these ArKO mice to establish whether the WDs degenerate in the male offspring at the same rate as that observed in the females.

Since the studies in *Tfm* mice and DBP + flutamide exposed rats (Chapter 6) raised the question of whether androgens alone are controlling WD development, pregnant rat dams were exposed to testosterone to investigate whether this was sufficient to stabilise the female WD or whether during normal male WD development, some other testicular factor may also be contributing (Chapter 7). Previous studies showed that exposing animals to exogenous maternal testosterone could masculinise the female reproductive tract, however there was considerable variation in the doses and timing of exposure reported to elicit this masculinisation with little information available detailing the phenotype of the rescued WD (section 1.5.3). In my studies, exposure to maternal testosterone (5 or 20 mgkg⁻¹) between e14.5-21.5 did not obviously affect male reproductive development but masculinised the female reproductive tract: in these females the WD was not only stabilised but some degree of differentiation was also initiated. Taken together with the results of the flutamide studies, these results confirm that testosterone alone is capable of rescuing the fetal WD and any other testicular factors are not essential for WD stabilisation if sufficient testosterone is available. However, the rescued female WD never differentiated as fully as the male WD,

possibly due to insufficient levels of testosterone or differences in testosterone delivery between normal males and testosterone-exposed females. Further studies could be undertaken exposing pregnant rats to higher concentrations of testosterone or to the more potent DHT to examine whether this can stimulate male-like morphological differentiation in the female WD. Furthermore, collaboration has recently been established with Dr. Matti Poutanen to examine WD development in female mice that over-express 17 β -HSD which results in increased androgen levels and genital masculinisation.

Previous studies have interfered with androgen action during the entire window of male reproductive development (e14-21), therefore studies were designed to investigate the precise window of androgen action in WD development by examining the effect of flutamide exposure either during the initial window of stabilisation (e14-17) or during morphological differentiation of the WD (e19-21) in the rat (Chapter 5). Surprisingly, exposure to flutamide between e19.5-21.5 did not obviously alter WD stabilisation or morphological differentiation. However, exposure to flutamide during the window of stabilisation inhibited WD morphological differentiation to the same extent as did exposure from e15.5-e21.5. This is in agreement with studies undertaken by Foster and Harris, which were published after my own studies had been undertaken, showing that exposure of pregnant rats to flutamide (50 mgkg⁻¹) on e16 or 17 resulted in missing epididymides in adulthood whereas exposure later in development (e18 or 19) only resulted in smaller epididymides (Foster and Harris, 2005). Furthermore, in my studies flutamide-exposure between e15.5-17.5 resulted in a similar high incidence of epididymal loss/abnormalities in late puberty and adulthood as did exposure throughout fetal reproductive development, highlighting that the pattern of WD coiling and its subsequent ability to develop fully during postnatal life is established early in fetal reproductive development (e15.5-17.5) when the AR is only expressed in the stromal cells. Care must be taken not to presume that androgens are not involved during the subsequent window of WD differentiation since exposure to this dose of flutamide is unlikely to be completely blocking androgen action. This could be further investigated in transgenic mice in which the AR could be ablated during defined windows in development thus allowing complete blockage of androgen action only during WD morphological differentiation. Alternatively, rats could be exposed only during this later window of WD morphological differentiation to a mixture of antiandrogenic compounds, which would reduce androgen action more completely than that induced by flutamide exposure alone.

Furthermore, these results also shed light on studies undertaken in the first year of this PhD, which are not reported in this thesis. An *in vitro* organ culture system was established in order to manipulate WD development in a defined environment. This culture system was

based on that reported by Tsuji and colleagues (1991) in which e15.5 male mouse urogenital ridges containing WDs and MDs were cultured for 4 days with or without gonads in serum-free medium. If the medium was supplemented with testosterone or DHT, coiling was induced in the future epididymal segment of the WD and the effects of exogenous androgens could be mimicked by co-culture with fetal testes. In my studies, WDs were recovered from rat fetuses at e18.5 or e19.5 and cultured for 3 or 4 days under similar conditions as used by Tsuji and colleagues. It was decided to use e18.5 WDs as this was prior to the onset of morphological differentiation (i.e. coiling) but after the regression of the male MD, therefore removing this as an extraneous factor. WDs could be maintained in this culture system for several days, however differentiation of these WDs could not be regulated by the addition of androgens or anti-androgens. For example, in serum- and androgen-free media, some WDs underwent considerable coiling while others collected from the same litter remained relatively straight. At the time, these results gave cause for concern and it was assumed that the culture system was not fully optimised. However, the studies detailed in chapter 5 of this thesis exposing dams to flutamide either during WD stabilisation or differentiation highlight that WD patterning is established by e17.5 therefore WDs recovered at e18.5 would already be patterned to differentiate and it is unlikely that this could be re-specified *in vitro*. Why some WDs from the same litter coiled and others did not remains somewhat of a mystery. Future studies could therefore utilise this *in vitro* culture system using WDs recovered at e15.5 or 16.5 to further manipulate and understand WD development. This would allow the study of not just androgen action in WD development but also the role for other factors that cannot be examined *in utero* as they could interfere with normal pregnancy and result in spontaneous abortion, such as growth factors.

A major puzzle arising from my flutamide studies reported in chapter 3 and 6 relates to why the majority of flutamide-exposed fetal males have WDs which are complete (11% incomplete at e21.5), but underdeveloped, whereas post-pubertally, in such animals the majority of WD-derived structures are largely absent (63-83% animals) with entire segments often missing. This data suggests that androgen action between e15.5-17.5 is essential to establish patterning of the fetal WD but how this patterning is maintained to enable differentiation into its different organ components, and the underlying molecular and biochemical pathways involved remain unknown. This information would not only increase our understanding of WD development but could also provide insight into how androgens might regulate the development and function of other organ systems such as the prostate. One obvious possible candidate for regulating WD patterning is the Hox gene family, which are steroid responsive and are known to play a role in regulating patterning and segmentation

of the reproductive tract (section 1.7.6.2). Investigations were undertaken to examine the role for Hox genes in normal and flutamide-exposed WD development during this thesis but no definitive results were gained, primarily due to technical difficulties. Commercial and in-house antibodies were used to localise Hox a10 and a11 in the rat WD, but these antibodies did not yield convincing immunostaining. Localisation was further investigated using in situ hybridisation but this was met with further complications as the mRNA sequence was not known for rat Hox a10 or 11 so the probe were designed based on mouse sequences. Like the immunohistochemistry, the in situ hybridisations did not provide conclusive results as staining was obtained from both the sense and anti-sense probes. Some Hox genes have been shown to have both sense and anti-sense transcripts therefore this data may be correct: probe specificity could be confirmed by performing a Northern blot prior to continuing these studies. Altered Hox gene expression may contribute to the flutamide-induced WD abnormalities therefore further investigation is required.

8.2 Cellular and molecular mechanisms in WD development

Studies were undertaken to investigate further the cellular and molecular effects of flutamide-exposure in the developing WD in order to gain insight into the mechanisms involved in WD patterning and development (Chapter 3 & 4). Somewhat surprisingly, flutamide exposure did not induce apoptosis in the male WD but rather impaired WD development by reducing cell proliferation in both the stromal and epithelial compartments. Furthermore, various extracellular matrices are expressed by cells in the developing WD including laminin, fibronectin and vimentin and exposure to flutamide interfered with their normal expression pattern and interrupted the basement membrane. The mechanisms involved in this ECM degradation are not yet fully understood but it is possible MMPs may play a role: this mechanism may contribute to the degradation of segments of the WD. These studies have not dramatically advanced our understanding of the molecular mechanisms involved in WD development but have provided some insight into the cellular mechanisms involved in normal and flutamide-perturbed WD development. These molecular mechanisms could be further interrogated using gene array studies, for example examining any differences in gene expression in the WDs from e16.5 flutamide-exposed male rats compared to those from age-matched controls.

8.3 Role for stromal-epithelial interactions in WD development

In common with many structures in the reproductive tract, simple columnar epithelial cells line the lumen of the WD with stromal cells surrounding this epithelium. In my studies, AR was first expressed in the stroma surrounding the WD at e15.5 but was not detected in the

epithelium until later, around e17.5. Throughout fetal life, this epithelial AR expression remained less intense than that observed in the stroma. It is therefore proposed that the stromal cells are the primary target for testosterone action and that testosterone regulates WD development by acting indirectly on the epithelium to induce its differentiation via stromal-epithelial interactions (Chapter 4). It became apparent that the epididymal epithelium does not fully differentiate until postnatal life, since p63 (an epithelial differentiation marker) was not expressed in the WD-derivatives until postnatal life, but that this was determined by androgen action during fetal life (Chapter 4). Conversely, stromal differentiation, as seen by smooth muscle actin expression, occurred during fetal life and was also impaired by flutamide exposure. This stromal effect was noted as early as e19.5, before any obvious signs of impaired epithelial development were apparent and before the epithelium underwent differentiation even in the control animals. This is consistent with previous studies in various organ systems which have shown that the stromal cells, via paracrine interactions, play a critical role in controlling epithelial proliferation, differentiation and development (section 1.7).

The mechanisms behind these paracrine interactions are poorly understood but modification of or, signalling via, the extracellular matrix and/or secretion of growth factors is likely to be involved. Flutamide-exposure did not alter AR expression highlighting that these WDs are still capable of AR-mediated androgen action. Reduced androgen action in flutamide-exposed WDs is likely to interfere with this paracrine signalling, and this may be one of the mechanisms involved in causing the flutamide-induced WD abnormalities seen in my studies. Further investigation into flutamide-induced impairment of these stromal-epithelial interactions is therefore required, especially the role for growth factors which have been highlighted to play a role in WD development as well as in other androgen-dependent processes. Previous studies have suggested a role for growth factors in reproductive tract development (Section 1.7.6.1) but the mechanisms underlying this are still unknown. Preliminary studies were undertaken during this thesis to investigate the role for EGF and IGF signalling pathways in WD development, however due to time constraints and technical limitations (namely availability of good antibodies for the rat) these studies did not yield any conclusive data and so needs to be repeated with new reagents. Since my studies have identified a more precise window of androgen action in the WD, the age to investigate their impact is now defined. As methods have been established to separate the stromal and epithelial compartments of various tissues (Cunha and Donjacour, 1987), primary WD cell lines could be utilised to manipulate gene expression/signalling *in vitro* in each cell compartment individually and so gain more insight into these stromal-epithelial interactions

in the WD. There is potential to use these cells for co-culture studies such as, for example, to investigate the effect of culturing AR negative stroma with control epithelium and examine the effect on cell proliferation and/or gene expression. It is worth mentioning that, in the past, stromal-epithelial co-culture experiments may not have provided hoped for breakthroughs in our understanding of androgen action in the reproductive tract, but this is a well-established technique that may yield some exciting results if utilised to examine genes known to be involved in androgen-dependent WD development. The existence of transgenic mice with a floxed AR gene (De Gendt *et al*, 2005) means that mice could be generated with cell specific ablation of the AR in each of the cellular compartments of the WD.

8.4 The role for DHT versus testosterone

Testosterone and DHT are known to mediate the development and differentiation of different structures in the male reproductive tract. This is unlikely to be due to different mechanisms of action but rather due to a difference in the availability of testosterone in the male reproductive tract since testosterone can mediate development of DHT-dependent tissues if it is present in high enough concentrations (Wilson and Lasnitzki, 1971; Wilson, 1996). DHT is a more potent androgen that dissociates less readily from the AR therefore activating androgen signalling for longer than testosterone does. At the onset of this thesis, I presumed that DHT-mediated tissues were more sensitive to the effects of androgen action and could therefore be more easily affected by anti-androgen action. However, this seems to be an oversimplification. The studies undertaken for this thesis and the evaluation of the literature has highlighted various discrepancies in the ability of androgens and anti-androgens to mediate testosterone- and DHT-dependent differentiation (summarised in Table 8.1). For example, CAH female fetuses or animals exposed to exogenous maternal testosterone are both able to masculinise DHT-dependent structures more readily than testosterone-dependent (WD) tissues (discussed in section 1.5). Both CAH females and female fetuses exposed to exogenous maternal testosterone depend on circulating testosterone, rather than testis-derived local testosterone as occurs naturally in males. This might explain why the WD is often not masculinised in these females as WD development appears to depend on high levels of androgen action, unlike DHT-dependent structures. My studies showed that if circulating testosterone levels are increase to sufficient concentrations, the WD can be rescued. This further suggests that WD stabilisation is a dose dependent event. Conversely, *Wnt4*^{-/-} female mice are exposed to local testosterone from the ovary, which can masculinise the WD but has no effect on the external genitalia (discussed in section 1.5.3.2). This raises the question of whether this difference is due to differences in the source of testosterone with WD development depending on local testosterone exposure while DHT-dependent structures

are masculinised by lower levels of circulating testosterone. However, this does not explain why in *Wnt4*^{-/-} female mice the levels of testosterone are capable of masculinising the WD but are not able to masculinise the external genitalia. This puzzle can be further developed using evidence from studies in which rats were exposed to various anti-androgens *in utero*. For example, DBP and Linuron both decrease testicular testosterone production and as a result, have a greater effect on WD-derived tissues than on DHT-dependent tissues (Mylchreest *et al*, 1998; Foster *et al*, 2000; Lambright *et al*, 2000; McIntyre *et al*, 2000; Foster *et al*, 2001; McIntyre *et al*, 2002). Conversely, flutamide and vinclozolin antagonise the AR but have no effect on local testosterone production and are able to impair masculinisation of DHT-dependent structures at lower doses than required to alter WD development. It is likely that testosterone is concentrated enough locally to compete with flutamide in the WD and so may explain why the WD is rescued in flutamide-exposed animals while the external genitalia are feminised. However, none of these studies provided definitive evidence for the differences in responsiveness of the different structures to testosterone or DHT, and it is not immediately obvious how this could be done. Studies would need to manipulate both the route of, as well as the dose of, androgen exposure in order to resolve this issue.

Table 8.1 Summary of ability of androgens and anti-androgens to alter development of testosterone- and DHT dependent structures.

Condition	Overall Phenotype	Mechanism	External genitalia	WD structures
AIS	Feminised male	AR inactive	Female	Male/female
Flutamide exposure	Feminised male	AR inactive	Female	Male/female
Linuron exposure	Feminised male	AR inactive (weak) and decreased testosterone	Male/female	Female
Vinclozolin exposure	Feminised male	AR inactive (weak)	Female	Male
DBP exposure	Feminised male	Decreased testosterone	Male/female	Female
5 α -reductase deficiency	Feminised male	No DHT	Female	Male
CAH	Masculinised female	Increased testosterone	Male	Female
PCOS	Masculinised female	Increased testosterone	Female	Female
Exogenous maternal testosterone	Masculinised female	Increased testosterone	Male	Male/female
Wnt 4 KO	Masculinised female	Increased testosterone	Female	Male

8.5 Conclusion

In conclusion, androgens alone appear to be sufficient for WD development, but in their absence a compensatory mechanism may try to rescue the male WD. WD differentiation is far more susceptible to blockade of androgen action than is its initial stabilisation and these effects may be mediated by disruption of stromal-epithelial interactions. These findings advance our understanding of WD development as for the first time they have identified the critical window for androgen action in the WD and thus have created an opportunity to study the elusive mechanisms behind androgen action. Since androgens act on many other systems in the body with many parallels existing between the male and female reproductive system, the outcomes of these studies may therefore impact on our understanding of WD development but also of other androgen-dependent processes such as the development, function and diseases of other reproductive tissues. It is interesting to note however, that over 25 years ago, Wilson and colleagues (1981) wondered how tissues develop the capacity early in fetal life to respond to hormonal stimulation later in development and how the same hormonal signal can be translated in different physiological effects in different tissues. Even though the results presented in this thesis advance our understanding of WD development, this question still remains largely unanswered today.

9 References

- Abbott, D. H., D. K. Barnett, C. M. Bruns and D. A. Dumesic (2005). "Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome?" *Hum Reprod Update* **11**(4): 357-74.
- Abbott, D. H., Dumesic, D.A., Eisner, J.R., Colman, R.J., and Kemnitz, J.W. (1998). "Insights into the development of PCOS from studies of prenatally androgenized female rhesus monkeys." *Trends in Endocrinology and Metabolism* **9**: 62-67.
- Achermann, J. C., M. Ito, P. C. Hindmarsh and J. L. Jameson (1999). "A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans." *Nat Genet* **22**(2): 125-6.
- Adham, I. M., J. M. Emmen and W. Engel (2000). "The role of the testicular factor INSL3 in establishing the gonadal position." *Mol Cell Endocrinol* **160**(1-2): 11-6.
- Ahmed, S. F., A. Cheng, L. Dovey, J. R. Hawkins, H. Martin, J. Rowland, N. Shimura, A. D. Tait and I. A. Hughes (2000). "Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome." *J Clin Endocrinol Metab* **85**(2): 658-65.
- Alarid, E. T., J. S. Rubin, P. Young, M. Chedid, D. Ron, S. A. Aaronson and G. R. Cunha (1994). "Keratinocyte growth factor functions in epithelial induction during seminal vesicle development." *Proc Natl Acad Sci U S A* **91**(3): 1074-8.
- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1994). *Molecular biology of the cell*. New York, Garland Publishing.
- Allard, S., P. Adin, L. Gouedard, N. di Clemente, N. Josso, M. C. Orgebin-Crist, J. Y. Picard and F. Xavier (2000). "Molecular mechanisms of hormone-mediated Mullerian duct regression: involvement of beta-catenin." *Development* **127**(15): 3349-60.
- Ammini, A. C., J. Pandey, M. Vijayaraghavan and U. Sabherwal (1994). "Human female phenotypic development: role of fetal ovaries." *J Clin Endocrinol Metab* **79**(2): 604-8.
- Andersson, S., D. M. Berman, E. P. Jenkins and D. W. Russell (1991). "Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism." *Nature* **354**(6349): 159-61.
- Andersson, S., R. W. Bishop and D. W. Russell (1989). "Expression cloning and regulation of steroid 5 alpha-reductase, an enzyme essential for male sexual differentiation." *J Biol Chem* **264**(27): 16249-55.
- Andersson, S. and D. W. Russell (1990). "Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases." *Proc Natl Acad Sci U S A* **87**(10): 3640-4.
- Arnold, J. T., D. G. Kaufman, M. Seppala and B. A. Lessey (2001). "Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model." *Hum Reprod* **16**(5): 836-45.
- Atanassova, N., C. McKinnell, J. Fisher and R. M. Sharpe (2005). "Neonatal treatment of rats with diethylstilboestrol (DES) induces stromal-epithelial abnormalities of the vas deferens and cauda epididymis in adulthood following delayed basal cell development." *Reproduction* **129**(5): 589-601.
- Atanassova, N., C. McKinnell, M. Walker, K. J. Turner, J. S. Fisher, M. Morley, M. R. Millar, N. P. Groome and R. M. Sharpe (1999). "Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood." *Endocrinology* **140**(11): 5364-73.
- Atanassova, N., C. McKinnell, K. Williams, K. J. Turner, J. S. Fisher, P. T. Saunders, M. R. Millar and R. M. Sharpe (2001). "Age-, cell- and region-specific immunoexpression of estrogen receptor alpha (but not estrogen receptor beta) during postnatal development of the epididymis and vas deferens of the rat and disruption of this pattern by neonatal treatment with diethylstilbestrol." *Endocrinology* **142**(2): 874-86.
- Attardi, B. and S. Ono (1974). "Cytosol androgen receptor from kidney of normal and testicular feminized (Tfm) mice." *Cell* **2**(4): 205-12.
- Aumailley, M. and N. Smyth (1998). "The role of laminins in basement membrane function." *J Anat* **193** (Pt 1): 1-21.
- Aupperle, H., D. Schoon and H. A. Schoon (2004). "Physiological and pathological expression of intermediate filaments in the equine endometrium." *Res Vet Sci* **76**(3): 249-55.

- Austin, H. B. (1995). "Dil analysis of cell migration during mullerian duct regression." *Dev Biol* **169**(1): 29-36.
- Avizienyte, E., A. W. Wyke, R. J. Jones, G. W. McLean, M. A. Westhoff, V. G. Brunton and M. C. Frame (2002). "Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling." *Nat Cell Biol* **4**(8): 632-8.
- Baker, T. G. (1963). "A Quantitative and Cytological Study of Germ Cells in Human Ovaries." *Proc R Soc Lond B Biol Sci* **158**: 417-33.
- Bale, P. M., N. J. Howard and J. E. Wright (1992). "Male pseudohermaphroditism in XY children with female phenotype." *Pediatr Pathol* **12**(1): 29-49.
- Bardoni, B., E. Zanaria, S. Guioli, G. Floridia, K. C. Worley, G. Tonini, E. Ferrante, G. Chiumello, E. R. McCabe, M. Fraccaro and et al. (1994). "A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal." *Nat Genet* **7**(4): 497-501.
- Barlow, N. J. and P. M. Foster (2003). "Pathogenesis of male reproductive tract lesions from gestation through adulthood following in utero exposure to Di(n-butyl) phthalate." *Toxicol Pathol* **31**(4): 397-410.
- Barrionuevo, F., S. Bagheri-Fam, J. Klattig, R. Kist, M. M. Taketo, C. Englert and G. Scherer (2006). "Homozygous inactivation of Sox9 causes complete XY sex reversal in mice." *Biol Reprod* **74**(1): 195-201.
- Barsoum, I. and H. H. Yao (2006). "The road to maleness: from testis to Wolffian duct." *Trends Endocrinol Metab* **17**(6): 223-8.
- Baum, M. J., P. J. Woutersen and A. K. Slob (1991). "Sex difference in whole-body androgen content in rats on fetal days 18 and 19 without evidence that androgen passes from males to females." *Biol Reprod* **44**(5): 747-51.
- Beato, M., M. Truss and S. Chavez (1996). "Control of transcription by steroid hormones." *Ann N Y Acad Sci* **784**: 93-123.
- Beitel, L. K., Y. A. Elhaji, R. Lumbroso, S. S. Wing, V. Panet-Raymond, B. Gottlieb, L. Pinsky and M. A. Trifiro (2002). "Cloning and characterization of an androgen receptor N-terminal-interacting protein with ubiquitin-protein ligase activity." *J Mol Endocrinol* **29**(1): 41-60.
- Benson, G. V., T. H. Nguyen and R. L. Maas (1995). "The expression pattern of the murine Hoxa-10 gene and the sequence recognition of its homeodomain reveal specific properties of Abdominal B-like genes." *Mol Cell Biol* **15**(3): 1591-601.
- Bentvelsen, F. M., A. O. Brinkmann, P. van der Schoot, J. E. van der Linden, T. H. van der Kwast, W. J. Boersma, F. H. Schroder and J. M. Nijman (1995). "Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat." *Mol Cell Endocrinol* **113**(2): 245-53.
- Bentvelsen, F. M., M. J. McPhaul, J. D. Wilson and F. W. George (1994). "The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen." *Mol Cell Endocrinol* **105**(1): 21-6.
- Berman, D. M. and D. W. Russell (1993). "Cell-type-specific expression of rat steroid 5 alpha-reductase isozymes." *Proc Natl Acad Sci U S A* **90**(20): 9359-63.
- Berman, D. M., H. Tian and D. W. Russell (1995). "Expression and regulation of steroid 5 alpha-reductase in the urogenital tract of the fetal rat." *Mol Endocrinol* **9**(11): 1561-70.
- Bernard, P. and V. R. Harley (2006). "Wnt4 action in gonadal development and sex determination." *Int J Biochem Cell Biol*.
- Berrevoets, C. A., P. Doesburg, K. Stekette, J. Trapman and A. O. Brinkmann (1998). "Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor2)." *Mol Endocrinol* **12**(8): 1172-83.
- Bevan, C. L., S. Hoare, F. Claessens, D. M. Heery and M. G. Parker (1999). "The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1." *Mol Cell Biol* **19**(12): 8383-92.
- Bigsby, R. M. (2002). "Control of growth and differentiation of the endometrium: the role of tissue interactions." *Ann N Y Acad Sci* **955**: 110-7; discussion 118, 396-406.
- Block, K., A. Kardana, P. Igarashi and H. S. Taylor (2000). "In utero diethylstilbestrol (DES) exposure alters Hox gene expression in the developing mullerian system." *Faseb J* **14**(9): 1101-8.
- Boehmer, A. L., O. Brinkmann, H. Bruggenwirth, C. van Assendelft, B. J. Otten, M. C. Verleun-Mooijman, M. F. Niermeijer, H. G. Brunner, C. W. Rouwe, J. J. Waelkens, W. Oostdijk, W.

- J. Kleijer, T. H. van der Kwast, M. A. de Vroede and S. L. Drop (2001). "Genotype versus phenotype in families with androgen insensitivity syndrome." *J Clin Endocrinol Metab* **86**(9): 4151-60.
- Bogan, J. S. and D. C. Page (1994). "Ovary? Testis?--A mammalian dilemma." *Cell* **76**(4): 603-7.
- Bok, G. and U. Drews (1983). "The role of the Wolffian ducts in the formation of the sinus vagina: an organ culture study." *J Embryol Exp Morphol* **73**: 275-95.
- Bomgardner, D., B. T. Hinton and T. T. Turner (2001). "Hox transcription factors may play a role in regulating segmental function of the adult epididymis." *J Androl* **22**(4): 527-31.
- Bomgardner, D., B. T. Hinton and T. T. Turner (2003). "5' hox genes and meis 1, a hox-DNA binding cofactor, are expressed in the adult mouse epididymis." *Biol Reprod* **68**(2): 644-50.
- Bossert, N. L., K. G. Nelson, K. A. Ross, T. Takahashi and J. A. McLachlan (1990). "Epidermal growth factor binding and receptor distribution in the mouse reproductive tract during development." *Dev Biol* **142**(1): 75-85.
- Bowman, C. J., N. J. Barlow, K. J. Turner, D. G. Wallace and P. M. Foster (2003). "Effects of in utero exposure to finasteride on androgen-dependent reproductive development in the male rat." *Toxicol Sci* **74**(2): 393-406.
- Bowman, C. J., K. J. Turner, M. Sar, N. J. Barlow, K. W. Gaido and P. M. Foster (2005). "Altered gene expression during rat Wolffian duct development following di(n-butyl) phthalate exposure." *Toxicol Sci* **86**(1): 161-74.
- Brennan, J. and B. Capel (2004). "One tissue, two fates: molecular genetic events that underlie testis versus ovary development." *Nat Rev Genet* **5**(7): 509-21.
- Brennan, J., J. Karl and B. Capel (2002). "Divergent vascular mechanisms downstream of Sry establish the arterial system in the XY gonad." *Dev Biol* **244**(2): 418-28.
- Brennan, J., C. Tilmann and B. Capel (2003). "Pdgrf-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad." *Genes Dev* **17**(6): 800-10.
- Brinkmann, A. (2003). Androgen Physiology; Receptor and Metabolic disorders. In: *Endocrinology of male reproduction*. R. McLachlan.
- Brinkmann, A. O. (2001). "Molecular basis of androgen insensitivity." *Mol Cell Endocrinol* **179**(1-2): 105-9.
- Brown, C. J., S. J. Goss, D. B. Lubahn, D. R. Joseph, E. M. Wilson, F. S. French and H. F. Willard (1989). "Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism." *Am J Hum Genet* **44**(2): 264-9.
- Buchanan, D. L., T. Kurita, J. A. Taylor, D. B. Lubahn, G. R. Cunha and P. S. Cooke (1998). "Role of stromal and epithelial estrogen receptors in vaginal epithelial proliferation, stratification, and cornification." *Endocrinology* **139**(10): 4345-52.
- Buehr, M., S. Gu and A. McLaren (1993). "Mesonephric contribution to testis differentiation in the fetal mouse." *Development* **117**(1): 273-81.
- Burgoyne, P. S. (1988). "Role of mammalian Y chromosome in sex determination." *Philos Trans R Soc Lond B Biol Sci* **322**(1208): 63-72.
- Byers, S., Jegou, B., MacCalman, C.D., Blaschuk, O.W. (1993). Sertoli cell adhesion molecules and the collective organization of the testis.
- . In: *The Sertoli Cell*. L. D. Russell, and Griswold, M.D. . Clearwater, Cache River Press: 461-476.
- Cain, M. P., S. A. Kramer, D. J. Tindall and D. A. Husmann (1994). "Alterations in maternal epidermal growth factor (EGF) effect testicular descent and epididymal development." *Urology* **43**(3): 375-8.
- Capel, B. (2000). "The battle of the sexes." *Mech Dev* **92**(1): 89-103.
- Carlsen, S. M., G. Jacobsen and P. Romundstad (2006). "Maternal testosterone levels during pregnancy are associated with offspring size at birth." *Eur J Endocrinol* **155**(2): 365-70.
- Carreau, S., C. Delalande, D. Silandre, S. Bourguiba and S. Lambard (2006). "Aromatase and estrogen receptors in male reproduction." *Mol Cell Endocrinol* **246**(1-2): 65-8.
- Carson-Jurica, M. A., W. T. Schrader and B. W. O'Malley (1990). "Steroid receptor family: structure and functions." *Endocr Rev* **11**(2): 201-20.
- Cattanach, B. M., C. E. Pollard and S. G. Hawker (1971). "Sex-reversed mice: XX and XO males." *Cytogenetics* **10**(5): 318-37.
- Chamberlain, N. L., E. D. Driver and R. L. Miesfeld (1994). "The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function." *Nucleic Acids Res* **22**(15): 3181-6.

- Chan, S. W. and J. H. Leatham (1975). "Placental steroidogenesis in the rat: progesterone production by tissue of the basal zone." *Endocrinology* **96**(2): 298-303.
- Chang, C. S., J. Kokontis and S. T. Liao (1988). "Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors." *Proc Natl Acad Sci U S A* **85**(19): 7211-5.
- Chang, W. Y., M. J. Wilson, L. Birch and G. S. Prins (1999). "Neonatal estrogen stimulates proliferation of periductal fibroblasts and alters the extracellular matrix composition in the rat prostate." *Endocrinology* **140**(1): 405-15.
- Choong, C. S., M. J. Sturm, J. A. Strophair, R. K. McCulloch, W. D. Tilley, P. J. Leedman and D. M. Hurley (1996). "Partial androgen insensitivity caused by an androgen receptor mutation at amino acid 907 (Gly-->Arg) that results in decreased ligand binding affinity and reduced androgen receptor messenger ribonucleic acid levels." *J Clin Endocrinol Metab* **81**(1): 236-43.
- Chua, S. S., Z. Q. Ma, L. Gong, S. H. Lin, F. J. DeMayo and S. Y. Tsai (2002). "Ectopic expression of FGF-3 results in abnormal prostate and Wolffian duct development." *Oncogene* **21**(12): 1899-908.
- Chung, L. W. and G. R. Cunha (1983). "Stromal-epithelial interactions: II. Regulation of prostatic growth by embryonic urogenital sinus mesenchyme." *Prostate* **4**(5): 503-11.
- Claessens, F., P. Alen, A. Devos, B. Peeters, G. Verhoeven and W. Rombauts (1996). "The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors." *J Biol Chem* **271**(32): 19013-6.
- Cook, J. C., L. S. Mullin, S. R. Frame and L. B. Biegel (1993). "Investigation of a mechanism for Leydig cell tumorigenesis by linuron in rats." *Toxicol Appl Pharmacol* **119**(2): 195-204.
- Cooke, G. M. and B. Robaire (1987). "The mechanism of rat epididymal 4-ene steroid 5 alpha-reductase." *J Steroid Biochem* **26**(3): 361-8.
- Cooke, P. S., D. L. Buchanan, P. Young, T. Setiawan, J. Brody, K. S. Korach, J. Taylor, D. B. Lubahn and G. R. Cunha (1997). "Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium." *Proc Natl Acad Sci U S A* **94**(12): 6535-40.
- Cooke, P. S., P. Young and G. R. Cunha (1991). "Androgen receptor expression in developing male reproductive organs." *Endocrinology* **128**(6): 2867-73.
- Cooke, P. S., P. Young, R. A. Hess and G. R. Cunha (1991). "Estrogen receptor expression in developing epididymis, efferent ductules, and other male reproductive organs." *Endocrinology* **128**(6): 2874-9.
- Couse, J. F. and K. S. Korach (1999). "Estrogen receptor null mice: what have we learned and where will they lead us?" *Endocr Rev* **20**(3): 358-417.
- Cunha, G. R. (1976). "Epithelial-stromal interactions in development of the urogenital tract." *Int Rev Cytol* **47**: 137-94.
- Cunha, G. R. (1976). "Stromal induction and specification of morphogenesis and cytodifferentiation of the epithelia of the Mullerian ducts and urogenital sinus during development of the uterus and vagina in mice." *J Exp Zool* **196**(3): 361-70.
- Cunha, G. R., E. T. Alarid, T. Turner, A. A. Donjacour, E. L. Boutin and B. A. Foster (1992). "Normal and abnormal development of the male urogenital tract. Role of androgens, mesenchymal-epithelial interactions, and growth factors." *J Androl* **13**(6): 465-75.
- Cunha, G. R., R. M. Bigsby, P. S. Cooke and Y. Sugimura (1985). "Stromal-epithelial interactions in adult organs." *Cell Differ* **17**(3): 137-48.
- Cunha, G. R. and L. W. Chung (1981). "Stromal-epithelial interactions--I. Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice." *J Steroid Biochem* **14**(12): 1317-24.
- Cunha, G. R., P. S. Cooke and T. Kurita (2004). "Role of stromal-epithelial interactions in hormonal responses." *Arch Histol Cytol* **67**(5): 417-34.
- Cunha, G. R. and A. Donjacour (1987). "Mesenchymal-epithelial interactions: technical considerations." *Prog Clin Biol Res* **239**: 273-82.
- Cunha, G. R. and A. Donjacour (1987). "Stromal-epithelial interactions in normal and abnormal prostatic development." *Prog Clin Biol Res* **239**: 251-72.
- Cunha, G. R., A. A. Donjacour, P. S. Cooke, S. Mee, R. M. Bigsby, S. J. Higgins and Y. Sugimura (1987). "The endocrinology and developmental biology of the prostate." *Endocr Rev* **8**(3): 338-62.

- Cunha, G. R., Donjacour, A.A., Hayward, S.W. (1998). Reciprocal mesenchymal-epithelial interactions in development of the male urogenital tract. In: Reproductive and developmental toxicology. K. S. Korach, Marcell Dekker Inc.: 509-530.
- Cunha, G. R., B. Foster, A. Thomson, Y. Sugimura, N. Tanji, M. Tsuji, N. Terada, P. W. Finch and A. A. Donjacour (1995). "Growth factors as mediators of androgen action during the development of the male urogenital tract." World J Urol **13**(5): 264-76.
- Cunha, G. R., H. Fujii, B. L. Neubauer, J. M. Shannon, L. Sawyer and B. A. Reese (1983). "Epithelial-mesenchymal interactions in prostatic development. I. morphological observations of prostatic induction by urogenital sinus mesenchyme in epithelium of the adult rodent urinary bladder." J Cell Biol **96**(6): 1662-70.
- Cunha, G. R., S. W. Hayward, R. Dahiya and B. A. Foster (1996). "Smooth muscle-epithelial interactions in normal and neoplastic prostatic development." Acta Anat (Basel) **155**(1): 63-72.
- Cunha, G. R., S. W. Hayward and Y. Z. Wang (2002). "Role of stroma in carcinogenesis of the prostate." Differentiation **70**(9-10): 473-85.
- Cunha, G. R. and P. Young (1991). "Inability of Tfm (testicular feminization) epithelial cells to express androgen-dependent seminal vesicle secretory proteins in chimeric tissue recombinants." Endocrinology **128**(6): 3293-8.
- Cunha, G. R. and P. Young (1992). "Role of stroma in oestrogen-induced epithelial proliferation." Epithelial Cell Biol **1**(1): 18-31.
- Cunha, G. R., P. Young, S. Hamamoto, R. Guzman and S. Nandi (1992). "Developmental response of adult mammary epithelial cells to various fetal and neonatal mesenchymes." Epithelial Cell Biol **1**(3): 105-18.
- Cunha, G. R., P. Young, Y. K. Hom, P. S. Cooke, J. A. Taylor and D. B. Lubahn (1997). "Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants." J Mammary Gland Biol Neoplasia **2**(4): 393-402.
- Danen, E. H. and K. M. Yamada (2001). "Fibronectin, integrins, and growth control." J Cell Physiol **189**(1): 1-13.
- De Felici, M., F. G. Klinger, D. Farini, M. L. Scaldaferri, S. Iona and M. Lobascio (2005). "Establishment of oocyte population in the fetal ovary: primordial germ cell proliferation and oocyte programmed cell death." Reprod Biomed Online **10**(2): 182-91.
- De Gendt, K., N. Atanassova, K. A. Tan, L. R. de Franca, G. G. Parreira, C. McKinnell, R. M. Sharpe, P. T. Saunders, J. I. Mason, S. Hartung, R. Ivell, E. Denolet and G. Verhoeven (2005). "Development and function of the adult generation of Leydig cells in mice with Sertoli cell-selective or total ablation of the androgen receptor." Endocrinology **146**(9): 4117-26.
- Dolle, P., J. C. Izpisua-Belmonte, J. M. Brown, C. Tickle and D. Duboule (1991). "HOX-4 genes and the morphogenesis of mammalian genitalia." Genes Dev **5**(10): 1767-7.
- Donahoe, P. K., Y. Ito, J. M. Price and W. H. Hendren, 3rd (1977). "Mullerian inhibiting substance activity in bovine fetal, newborn and prepubertal testes." Biol Reprod **16**(2): 238-43.
- Donjacour, A. A. and G. R. Cunha (1991). "Stromal regulation of epithelial function." Cancer Treat Res **53**: 335-64.
- Drews, U. (1975). "Direct and mediated effects of testosterone: the development of intersexes in sex reversed mosaic mice, heterozygous for testicular feminization." Anat Embryol (Berl) **146**(3): 325-40.
- Drews, U. (2000). "Local mechanisms in sex specific morphogenesis." Cytogenet Cell Genet **91**(1-4): 72-80.
- Drews, U. and V. Alonso-Lozano (1974). "X-inactivation pattern in the epididymis of sex-reversed mice heterozygous for testicular feminization." J Embryol Exp Morphol **32**(1): 217-25.
- Drews, U. and H. J. Dieterich (1978). "Cell death in the mosaic epididymis of sex reversed mice, heterozygous for testicular feminization." Anat Embryol (Berl) **152**(2): 193-203.
- Du, H. and H. S. Taylor (2004). "Molecular regulation of mullerian development by Hox genes." Ann N Y Acad Sci **1034**: 152-65.
- Dyche, W. J. (1979). "A comparative study of the differentiation and involution of the Mullerian duct and Wolffian duct in the male and female fetal mouse." J Morphol **162**(2): 175-209.
- Dziadek, M. and R. Timpl (1985). "Expression of nidogen and laminin in basement membranes during mouse embryogenesis and in teratocarcinoma cells." Dev Biol **111**(2): 372-82.

- Eddy, E. M., T. F. Washburn, D. O. Bunch, E. H. Goulding, B. C. Gladen, D. B. Lubahn and K. S. Korach (1996). "Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility." *Endocrinology* **137**(11): 4796-805.
- Edwards, T. M., B. C. Moore and L. J. Guillette, Jr. (2006). "Reproductive dysgenesis in wildlife: a comparative view." *Int J Androl* **29**(1): 109-21.
- El-Gehani, F., F. P. Zhang, P. Pakarinen, A. Rannikko and I. Huhtaniemi (1998). "Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis." *Biol Reprod* **58**(1): 116-23.
- Ema, M., A. Harazona, E. Miyawaki and Y. Ogawa (1997). "Embryo lethality following maternal exposure to dibutyl phthalate during early pregnancy in rats." *Bull Environ Contam Toxicol* **58**(4): 636-43.
- Emmen, J. M., A. McLuskey, I. M. Adham, W. Engel, J. A. Grootegoed and A. O. Brinkmann (2000). "Hormonal control of gubernaculum development during testis descent: gubernaculum outgrowth in vitro requires both insulin-like factor and androgen." *Endocrinology* **141**(12): 4720-7.
- Erickson, A. C. and J. R. Couchman (2000). "Still more complexity in mammalian basement membranes." *J Histochem Cytochem* **48**(10): 1291-306.
- Evans, R. M. (1988). "The steroid and thyroid hormone receptor superfamily." *Science* **240**(4854): 889-95.
- Faber, P. W., H. C. van Rooij, H. A. van der Korput, W. M. Baarends, A. O. Brinkmann, J. A. Grootegoed and J. Trapman (1991). "Characterization of the human androgen receptor transcription unit." *J Biol Chem* **266**(17): 10743-9.
- Faiman, C., Winter, J. S. D., Reyes, F.I. (1981). *Endocrinology of the Fetal Testis*. New York, Raven Press.
- Favier, B. and P. Dolle (1997). "Developmental functions of mammalian Hox genes." *Mol Hum Reprod* **3**(2): 115-31.
- Ferdous, A., F. Gonzalez, L. Sun, T. Kodadek and S. A. Johnston (2001). "The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II." *Mol Cell* **7**(5): 981-91.
- Fisher, C. R., K. H. Graves, A. F. Parlow and E. R. Simpson (1998). "Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene." *Proc Natl Acad Sci U S A* **95**(12): 6965-70.
- Fisher, J. S., S. Macpherson, N. Marchetti and R. M. Sharpe (2003). "Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate." *Hum Reprod* **18**(7): 1383-94.
- Foster, P. M., R. C. Cattley and E. Mylchreest (2000). "Effects of di-n-butyl phthalate (DBP) on male reproductive development in the rat: implications for human risk assessment." *Food Chem Toxicol* **38**(1 Suppl): S97-9.
- Foster, P. M. and M. W. Harris (2005). "Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages." *Toxicol Sci* **85**(2): 1024-32.
- Foster, P. M., E. Mylchreest, K. W. Gaido and M. Sar (2001). "Effects of phthalate esters on the developing reproductive tract of male rats." *Hum Reprod Update* **7**(3): 231-5.
- Foster, P. M., L. V. Thomas, M. W. Cook and S. D. Gangolli (1980). "Study of the testicular effects and changes in zinc excretion produced by some n-alkyl phthalates in the rat." *Toxicol Appl Pharmacol* **54**(3): 392-8.
- Freedman, L. P. (1992). "Anatomy of the steroid receptor zinc finger region." *Endocr Rev* **13**(2): 129-45.
- Frisch, S. M. and H. Francis (1994). "Disruption of epithelial cell-matrix interactions induces apoptosis." *J Cell Biol* **124**(4): 619-26.
- Fujieda, K., C. Faiman, F. I. Reyes and J. S. Winter (1981). "The control of steroidogenesis by human fetal adrenal cells in tissue culture. III. The effects of various hormonal peptides." *J Clin Endocrinol Metab* **53**(4): 690-3.
- Gehring, U. and G. M. Tomkins (1974). "Characterization of a hormone receptor defect in the androgen-insensitivity mutant." *Cell* **3**(1): 59-64.
- Gelmann, E. P. (2002). "Molecular biology of the androgen receptor." *J Clin Oncol* **20**(13): 3001-15.
- Georgatsou, E., P. Bourgarel and T. Meo (1993). "Male-specific expression of mouse sex-limited protein requires growth hormone, not testosterone." *Proc Natl Acad Sci U S A* **90**(8): 3626-30.

- George, F. W. (1997). "Androgen metabolism in the prostate of the finasteride-treated, adult rat: a possible explanation for the differential action of testosterone and 5 alpha-dihydrotestosterone during development of the male urogenital tract." *Endocrinology* **138**(3): 871-7.
- George, F. W., and Wilson, J. (1994). Gonads and ducts in mammals. In: *The Physiology of Reproduction*. E. Knobil, and Neill, J.D. New York, Raven Press.
- George, F. W. and J. D. Wilson (1978). "Conversion of androgen to estrogen by the human fetal ovary." *J Clin Endocrinol Metab* **47**(3): 550-5.
- Gibori, G. and R. Sridaran (1981). "Sites of androgen and estradiol production in the second half of pregnancy in the rat." *Biol Reprod* **24**(2): 249-56.
- Gilbert, S. F. (2003). *Developmental Biology*. Sunderland, MA, Sinauer Associates, Inc.
- Ginsburg, M., M. H. Snow and A. McLaren (1990). "Primordial germ cells in the mouse embryo during gastrulation." *Development* **110**(2): 521-8.
- Gioeli, D., S. B. Ficarro, J. J. Kwiek, D. Aaronson, M. Hancock, A. D. Catling, F. M. White, R. E. Christian, R. E. Settlege, J. Shabanowitz, D. F. Hunt and M. J. Weber (2002). "Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites." *J Biol Chem* **277**(32): 29304-14.
- Glass, C. K. and M. G. Rosenfeld (2000). "The coregulator exchange in transcriptional functions of nuclear receptors." *Genes Dev* **14**(2): 121-41.
- Gobinet, J., N. Poujol and C. Sultan (2002). "Molecular action of androgens." *Mol Cell Endocrinol* **198**(1-2): 15-24.
- Goffin, F., F. Frankenke, A. Beliard, S. Perrier D'Hauterive, M. R. Pignon, V. Geenen and J. M. Foidart (2002). "Human endometrial epithelial cells modulate the activation of gelatinase a by stromal cells." *Gynecol Obstet Invest* **53**(2): 105-11.
- Golsteyn, E. J., J. S. Graham, H. J. Goren and Y. A. Lefebvre (1989). "Phosphorylation status of nuclear and cytosolic androgen receptors in the rat ventral prostate." *Prostate* **14**(2): 91-101.
- Gottlieb, B., L. K. Beitel, R. Lumbroso, L. Pinsky and M. Trifiro (1999). "Update of the androgen receptor gene mutations database." *Hum Mutat* **14**(2): 103-14.
- Gray, H. (1918, 2000). "Anatomy of the Human Body." Retrieved 27/10/2006, 2006, from www.bartleby.com/107/271006.
- Gray, L. E., Jr., J. S. Ostby and W. R. Kelce (1994). "Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat." *Toxicol Appl Pharmacol* **129**(1): 46-52.
- Gray, L. E., Jr., C. Wolf, C. Lambright, P. Mann, M. Price, R. L. Cooper and J. Ostby (1999). "Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat." *Toxicol Ind Health* **15**(1-2): 94-118.
- Gray, L. E., J. Ostby, J. Furr, C. J. Wolf, C. Lambright, L. Parks, D. N. Veeramachaneni, V. Wilson, M. Price, A. Hotchkiss, E. Orlando and L. Guillette (2001). "Effects of environmental antiandrogens on reproductive development in experimental animals." *Hum Reprod Update* **7**(3): 248-64.
- Greene, R. R., Burrill, M.W., and Ivy, A.C. (1939). "Experimental intersexuality: The effect of antenatal androgens on sexual development of female rats." *Am. J. Anat* **65**: 415-469.
- Gregory, C. W., X. Fei, L. A. Ponguta, B. He, H. M. Bill, F. S. French and E. M. Wilson (2004). "Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer." *J Biol Chem* **279**(8): 7119-30.
- Grino, P. B., J. E. Griffin and J. D. Wilson (1987). "Transformation of the androgen receptor to the deoxyribonucleic acid-binding state: studies in homogenates and intact cells." *Endocrinology* **120**(5): 1914-20.
- Grino, P. B., J. E. Griffin and J. D. Wilson (1990). "Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone." *Endocrinology* **126**(2): 1165-72.
- Grishkovskaya, I., G. V. Avvakumov, G. Sklenar, D. Dales, G. L. Hammond and Y. A. Muller (2000). "Crystal structure of human sex hormone-binding globulin: steroid transport by a laminin G-like domain." *Embo J* **19**(4): 504-12.

- Gubbay, J., J. Collignon, P. Koopman, B. Capel, A. Economou, A. Munsterberg, N. Vivian, P. Goodfellow and R. Lovell-Badge (1990). "A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes." *Nature* **346**(6281): 245-50.
- Guo, W., T. P. Burris, Y. H. Zhang, B. L. Huang, J. Mason, K. C. Copeland, S. R. Kupfer, R. A. Pagon and E. R. McCabe (1996). "Genomic sequence of the DAX1 gene: an orphan nuclear receptor responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism." *J Clin Endocrinol Metab* **81**(7): 2481-6.
- Gupta, C. (1996). "The role of epidermal growth factor receptor (EGFR) in male reproductive tract differentiation: stimulation of EGFR expression and inhibition of Wolffian duct differentiation with anti-EGFR antibody." *Endocrinology* **137**(3): 905-10.
- Gupta, C. (1997). "Cellular localization of immunoreactive epidermal growth factor during Wolffian duct differentiation of the fetal mouse." *Urol Res* **25**(4): 277-81.
- Gupta, C., A. Chandorkar and A. P. Nguyen (1996). "Activation of androgen receptor in epidermal growth factor modulation of fetal mouse sexual differentiation." *Mol Cell Endocrinol* **123**(1): 89-95.
- Gupta, C. and J. Jaumotte (1993). "Epidermal growth factor binding in the developing male reproductive duct and its regulation by testosterone." *Endocrinology* **133**(4): 1778-82.
- Gupta, C., S. Siegel and D. Ellis (1991). "The role of EGF in testosterone-induced reproductive tract differentiation." *Dev Biol* **146**(1): 106-16.
- Gupta, C. and M. Singh (1996). "Stimulation of epidermal growth factor gene expression during the fetal mouse reproductive tract differentiation: role of androgen and its receptor." *Endocrinology* **137**(2): 705-11.
- Habert, R., H. Lejeune and J. M. Saez (2001). "Origin, differentiation and regulation of fetal and adult Leydig cells." *Mol Cell Endocrinol* **179**(1-2): 47-74.
- Habert, R. and R. Picon (1984). "Testosterone, dihydrotestosterone and estradiol-17 beta levels in maternal and fetal plasma and in fetal testes in the rat." *J Steroid Biochem* **21**(2): 193-8.
- Habert, R., V. Rouiller-Fabre, L. Lecerf, C. Levacher and J. M. Saez (1992). "Developmental changes in testosterone production by the rat testis in vitro during late fetal life." *Arch Androl* **29**(2): 191-7.
- Haider, S. G. (2004). "Cell biology of Leydig cells in the testis." *Int Rev Cytol* **233**: 181-241.
- Handelsman, D. J. (2003). Androgens. In: *Endocrinology of Male Reproduction*. R. McLachlan.
- Hannema, S. E., C. G. Print, D. S. Charnock-Jones, N. Coleman and I. A. Hughes (2006). "Changes in gene expression during Wolffian duct development." *Horm Res* **65**(4): 200-9.
- Hannema, S. E., I. S. Scott, J. Hodapp, H. Martin, N. Coleman, J. W. Schwabe and I. A. Hughes (2004). "Residual activity of mutant androgen receptors explains wolffian duct development in the complete androgen insensitivity syndrome." *J Clin Endocrinol Metab* **89**(11): 5815-22.
- Hannema, S. E., I. S. Scott, E. Rajpert-De Meyts, N. E. Skakkebaek, N. Coleman and I. A. Hughes (2006). "Testicular development in the complete androgen insensitivity syndrome." *J Pathol* **208**(4): 518-27.
- Hawkins, J. R., A. Taylor, P. Berta, J. Levilliers, B. Van der Auwera and P. N. Goodfellow (1992). "Mutational analysis of SRY: nonsense and missense mutations in XY sex reversal." *Hum Genet* **88**(4): 471-4.
- Hay, E. D. (1995). "An overview of epithelio-mesenchymal transformation." *Acta Anat (Basel)* **154**(1): 8-20.
- Hayashi, T., A. Yoshinaga, R. Ohno, N. Ishii, S. Kamata and T. Yamada (2004). "Expression of the p63 and Notch signaling systems in rat testes during postnatal development: comparison with their expression levels in the epididymis and vas deferens." *J Androl* **25**(5): 692-8.
- Hayward, S. W., L. S. Baskin, P. C. Haughney, B. A. Foster, A. R. Cunha, R. Dahiya, G. S. Prins and G. R. Cunha (1996). "Stromal development in the ventral prostate, anterior prostate and seminal vesicle of the rat." *Acta Anat (Basel)* **155**(2): 94-103.
- He, W. W., M. V. Kumar and D. J. Tindall (1991). "A frame-shift mutation in the androgen receptor gene causes complete androgen insensitivity in the testicular-feminized mouse." *Nucleic Acids Res* **19**(9): 2373-8.
- Hedger, M. P. and D. M. de Kretser (2000). "Leydig cell function and its regulation." *Results Probl Cell Differ* **28**: 69-110.
- Heikkila, M., H. Peltoketo and S. Vainio (2001). "Wnts and the female reproductive system." *J Exp Zool* **290**(6): 616-23.

- Heikkila, M., R. Prunskaitė, F. Naillat, P. Itaranta, J. Vuoristo, J. Leppaluoto, H. Peltoketo and S. Vainio (2005). "The partial female to male sex reversal in Wnt-4-deficient females involves induced expression of testosterone biosynthetic genes and testosterone production, and depends on androgen action." *Endocrinology* **146**(9): 4016-23.
- Heinlein, C. A. and C. Chang (2002). "Androgen receptor (AR) coregulators: an overview." *Endocr Rev* **23**(2): 175-200.
- Hess, R. A. (2000). "Oestrogen in fluid transport in efferent ducts of the male reproductive tract." *Rev Reprod* **5**(2): 84-92.
- Hess, R. A., D. Bunick and J. Bahr (2001). "Oestrogen, its receptors and function in the male reproductive tract - a review." *Mol Cell Endocrinol* **178**(1-2): 29-38.
- Hess, R. A., D. Bunick, K. H. Lee, J. Bahr, J. A. Taylor, K. S. Korach and D. B. Lubahn (1997). "A role for oestrogens in the male reproductive system." *Nature* **390**(6659): 509-12.
- Hess, R. A., D. Bunick, D. B. Lubahn, Q. Zhou and J. Bouma (2000). "Morphologic changes in efferent ductules and epididymis in estrogen receptor-alpha knockout mice." *J Androl* **21**(1): 107-21.
- Higgins, S. J., P. Young and G. R. Cunha (1989). "Induction of functional cytodifferentiation in the epithelium of tissue recombinants. II. Instructive induction of Wolffian duct epithelia by neonatal seminal vesicle mesenchyme." *Development* **106**(2): 235-50.
- Hiort, O. and P. M. Holterhus (2000). "The molecular basis of male sexual differentiation." *Eur J Endocrinol* **142**(2): 101-10.
- Hiort, O., Q. Huang, G. H. Sinnecker, A. Sadeghi-Nejad, K. Kruse, H. J. Wolfe and D. W. Yandell (1993). "Single strand conformation polymorphism analysis of androgen receptor gene mutations in patients with androgen insensitivity syndromes: application for diagnosis, genetic counseling, and therapy." *J Clin Endocrinol Metab* **77**(1): 262-6.
- Hiort, O., G. H. Sinnecker, P. M. Holterhus, E. M. Nitsche and K. Kruse (1996). "The clinical and molecular spectrum of androgen insensitivity syndromes." *Am J Med Genet* **63**(1): 218-22.
- Hoeck, W., S. Rusconi and B. Groner (1989). "Down-regulation and phosphorylation of glucocorticoid receptors in cultured cells. Investigations with a monospecific antiserum against a bacterially expressed receptor fragment." *J Biol Chem* **264**(24): 14396-402.
- Holmes, N. M., W. L. Miller and L. S. Baskin (2004). "Lack of defects in androgen production in children with hypospadias." *J Clin Endocrinol Metab* **89**(6): 2811-6.
- Horton, R., V. Pasupuletti and I. Antonipillai (1993). "Androgen induction of steroid 5 alpha-reductase may be mediated via insulin-like growth factor-I." *Endocrinology* **133**(2): 447-51.
- Hotchkiss, A. K., J. S. Ostby, J. G. Vandenberg and L. E. Gray, Jr. (2002). "Androgens and environmental antiandrogens affect reproductive development and play behavior in the Sprague-Dawley rat." *Environ Health Perspect* **110 Suppl 3**: 435-9.
- Hsieh-Li, H. M., D. P. Witte, M. Weinstein, W. Branford, H. Li, K. Small and S. S. Potter (1995). "Hoxa 11 structure, extensive antisense transcription, and function in male and female fertility." *Development* **121**(5): 1373-85.
- Hughes, I. A. (1997). Disorders of sexual development. In: *Molecular genetics of endocrine disorders*. R. V. Thakker. London, Chapman & Hall: 881-882.
- Hughes, I. A. and B. A. Evans (1987). "Androgen insensitivity in forty-nine patients: classification based on clinical and androgen receptor phenotypes." *Horm Res* **28**(1): 25-9.
- Hughes, I. A. and M. N. Patterson (1994). "Prenatal diagnosis of androgen insensitivity." *Clin Endocrinol (Oxf)* **40**(3): 295-6.
- Huhtaniemi, I. (1994). "Fetal testis--a very special endocrine organ." *Eur J Endocrinol* **130**(1): 25-31.
- Huhtaniemi, I. and J. Toppari (1995). "Endocrine, paracrine and autocrine regulation of testicular steroidogenesis." *Adv Exp Med Biol* **377**: 33-54.
- Hutson, J. M., S. Hasthorpe and C. F. Heyns (1997). "Anatomical and functional aspects of testicular descent and cryptorchidism." *Endocr Rev* **18**(2): 259-80.
- Ikawa, H., R. L. Trelstad, J. M. Hutson, T. F. Manganaro and P. K. Donahoe (1984). "Changing patterns of fibronectin, laminin, type IV collagen, and a basement membrane proteoglycan during rat Mullerian duct regression." *Dev Biol* **102**(1): 260-3.
- Imperato-McGinley, J. (2002). "5alpha-reductase-2 deficiency and complete androgen insensitivity: lessons from nature." *Adv Exp Med Biol* **511**: 121-31; discussion 131-4.
- Imperato-McGinley, J., L. Guerrero, T. Gautier and R. E. Peterson (1974). "Steroid 5alpha-reductase deficiency in man: an inherited form of male pseudohermaphroditism." *Science* **186**(4170): 1213-5.

- Imperato-McGinley, J., R. E. Peterson, T. Gautier, G. Cooper, R. Danner, A. Arthur, P. L. Morris, W. J. Sweeney and C. Shackleton (1982). "Hormonal evaluation of a large kindred with complete androgen insensitivity: evidence for secondary 5 alpha-reductase deficiency." *J Clin Endocrinol Metab* **54**(5): 931-41.
- Imperato-McGinley, J., R. S. Sanchez, J. R. Spencer, B. Yee and E. D. Vaughan (1992). "Comparison of the effects of the 5 alpha-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies." *Endocrinology* **131**(3): 1149-56.
- Imperato-McGinley, J. and Y. S. Zhu (2002). "Androgens and male physiology the syndrome of 5alpha-reductase-2 deficiency." *Mol Cell Endocrinol* **198**(1-2): 51-9.
- Ince, T. A., A. P. Cviko, B. J. Quade, A. Yang, F. D. McKeon, G. L. Mutter and C. P. Crum (2002). "p63 Coordinates anogenital modeling and epithelial cell differentiation in the developing female urogenital tract." *Am J Pathol* **161**(4): 1111-7.
- Inomata, T., Y. Eguchi and T. Nakamura (1989). "Origin of mullerian duct and its later development in relation to Wolffian duct and anogenital distance in the rat." *Nippon Juigaku Zasshi* **51**(4): 693-701.
- Jaffe, R. B. (1969). "Testosterone metabolism in target tissues. Hypothalamic and pituitary tissues of the adult rat and human fetus, and the immature rat epiphysis." *Steroids* **14**(5): 483-98.
- Jaworski, T. (2006). "Degradation and beyond: Control of androgen receptor activity by the proteasome system." *Cell Mol Biol Lett* **11**(1): 109-31.
- Jenster, G., J. Trapman and A. O. Brinkmann (1993). "Nuclear import of the human androgen receptor." *Biochem J* **293** (Pt 3): 761-8.
- Jirsova, Z. and Z. Vernerova (1993). "Involution of the Wolffian duct in the rat." *Funct Dev Morphol* **3**(3): 205-6.
- Johnson, G. R., B. Kannan, M. Shoyab and K. Stromberg (1993). "Amphiregulin induces tyrosine phosphorylation of the epidermal growth factor receptor and p185erbB2. Evidence that amphiregulin acts exclusively through the epidermal growth factor receptor at the surface of human epithelial cells." *J Biol Chem* **268**(4): 2924-31.
- Jones, N. (1990). "Transcriptional regulation by dimerization: two sides to an incestuous relationship." *Cell* **61**(1): 9-11.
- Jones, R. D., P. J. Pugh, J. Hall, K. S. Channer and T. H. Jones (2003). "Altered circulating hormone levels, endothelial function and vascular reactivity in the testicular feminised mouse." *Eur J Endocrinol* **148**(1): 111-20.
- Jordan, B. K., M. Mohammed, S. T. Ching, E. Delot, X. N. Chen, P. Dewing, A. Swain, P. N. Rao, B. R. Elejalde and E. Vilain (2001). "Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans." *Am J Hum Genet* **68**(5): 1102-9.
- Josso, N., N. di Clemente and L. Gouedard (2001). "Anti-Mullerian hormone and its receptors." *Mol Cell Endocrinol* **179**(1-2): 25-32.
- Jost, A. (1972). "A new look at the mechanisms controlling sex differentiation in mammals." *Johns Hopkins Med J* **130**(1): 38-53.
- Jost, A., P. Gonsse-Danysz and R. Jacquot (1953). "[Studies on physiology of fetal hypophysis in rabbits and its relation to testicular function.]." *J Physiol (Paris)* **45**(1): 134-6.
- Jost, A., B. Vigier, J. Prepin and J. P. Perchellet (1973). "Studies on sex differentiation in mammals." *Recent Prog Horm Res* **29**: 1-41.
- Kanai, Y. and P. Koopman (1999). "Structural and functional characterization of the mouse Sox9 promoter: implications for campomelic dysplasia." *Hum Mol Genet* **8**(4): 691-6.
- Kang, Y. and J. Massague (2004). "Epithelial-mesenchymal transitions: twist in development and metastasis." *Cell* **118**(3): 277-9.
- Kang, Z., A. Pirskanen, O. A. Janne and J. J. Palvimo (2002). "Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex." *J Biol Chem* **277**(50): 48366-71.
- Kashimata, M., M. Hiramatsu and N. Minami (1988). "Sex difference in epidermal growth factor receptor levels in rat liver plasma membrane." *Endocrinology* **122**(5): 1707-14.
- Katchen, B. and S. Buxbaum (1975). "Disposition of a new, nonsteroid, antiandrogen, alpha, alpha, alpha-trifluoro-2-methyl-4'-nitro-m-propionotoluidide (Flutamide), in men following a single oral 200 mg dose." *J Clin Endocrinol Metab* **41**(2): 373-9.
- Kaufman, M. H. (1992). *The atlas of mouse development*. London, Academic Press.

- Keating, R. J. and R. K. Tcholakian (1983). "In vivo patterns of circulating testosterone following castration and intramuscular testosterone propionate injections of adult male rats." *Steroids* **42**(1): 63-76.
- Keenan, B. S., W. J. Meyer, 3rd, A. J. Hadjian, H. W. Jones and C. J. Migeon (1974). "Syndrome of androgen insensitivity in man: absence of 5 alpha-dihydrotestosterone binding protein in skin fibroblasts." *J Clin Endocrinol Metab* **38**(6): 1143-6.
- Kelce, W. R., C. R. Lambright, L. E. Gray, Jr. and K. P. Roberts (1997). "Vinclozolin and p,p'-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism." *Toxicol Appl Pharmacol* **142**(1): 192-200.
- Kelce, W. R., E. Monosson, M. P. Gamcsik, S. C. Laws and L. E. Gray, Jr. (1994). "Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites." *Toxicol Appl Pharmacol* **126**(2): 276-85.
- Kemppainen, J. A., M. V. Lane, M. Sar and E. M. Wilson (1992). "Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones." *J Biol Chem* **267**(2): 968-74.
- Kim, Y., A. Kobayashi, R. Sekido, L. DiNapoli, J. Brennan, M. C. Chaboissier, F. Poulat, R. R. Behringer, R. Lovell-Badge and B. Capel (2006). "Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination." *PLoS Biol* **4**(6): e187.
- Kishimoto, M., R. Fujiki, S. Takezawa, Y. Sasaki, T. Nakamura, K. Yamaoka, H. Kitagawa and S. Kato (2006). "Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications." *Endocr J* **53**(2): 157-72.
- Kitamura, K., M. Yanazawa, N. Sugiyama, H. Miura, A. Iizuka-Kogo, M. Kusaka, K. Omichi, R. Suzuki, Y. Kato-Fukui, K. Kamiirisa, M. Matsuo, S. Kamijo, M. Kasahara, H. Yoshioka, T. Ogata, T. Fukuda, I. Kondo, M. Kato, W. B. Dobyns, M. Yokoyama and K. Morohashi (2002). "Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans." *Nat Genet* **32**(3): 359-69.
- Koopman, P., J. Gubbay, N. Vivian, P. Goodfellow and R. Lovell-Badge (1991). "Male development of chromosomally female mice transgenic for Sry." *Nature* **351**(6322): 117-21.
- Krege, J. H., J. B. Hodgin, J. F. Couse, E. Enmark, M. Warner, J. F. Mahler, M. Sar, K. S. Korach, J. A. Gustafsson and O. Smithies (1998). "Generation and reproductive phenotypes of mice lacking estrogen receptor beta." *Proc Natl Acad Sci U S A* **95**(26): 15677-82.
- Kuiper, G. G., P. E. De Ruiter and A. O. Brinkmann (1993). "Androgen receptor phosphorylation." *Ann N Y Acad Sci* **684**: 224-6.
- Kuiper, G. G., E. Enmark, M. Peltö-Huikko, S. Nilsson and J. A. Gustafsson (1996). "Cloning of a novel receptor expressed in rat prostate and ovary." *Proc Natl Acad Sci U S A* **93**(12): 5925-30.
- Kumagai, J., S. Y. Hsu, H. Matsumi, J. S. Roh, P. Fu, J. D. Wade, R. A. Bathgate and A. J. Hsueh (2002). "INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent." *J Biol Chem* **277**(35): 31283-6.
- Kurita, T., P. S. Cooke and G. R. Cunha (2001). "Epithelial-stromal tissue interaction in paramesonephric (Mullerian) epithelial differentiation." *Dev Biol* **240**(1): 194-211.
- Kurita, T. and G. R. Cunha (2001). "Roles of p63 in differentiation of Mullerian duct epithelial cells." *Ann N Y Acad Sci* **948**: 9-12.
- Kurita, T., A. A. Mills and G. R. Cunha (2004). "Roles of p63 in the diethylstilbestrol-induced cervicovaginal adenosis." *Development* **131**(7): 1639-49.
- Kurita, T., P. Young, J. R. Brody, J. P. Lydon, B. W. O'Malley and G. R. Cunha (1998). "Stromal progesterone receptors mediate the inhibitory effects of progesterone on estrogen-induced uterine epithelial cell deoxyribonucleic acid synthesis." *Endocrinology* **139**(11): 4708-13.
- Labrie, F., Y. Sugimoto, V. Luu-The, J. Simard, Y. Lachance, D. Bachvarov, G. Leblanc, F. Durocher and N. Paquet (1992). "Structure of human type II 5 alpha-reductase gene." *Endocrinology* **131**(3): 1571-3.
- Lamb, J. C., H. English, P. L. Levandoski, G. R. Rhodes, R. K. Johnson and J. T. Isaacs (1992). "Prostatic involution in rats induced by a novel 5 alpha-reductase inhibitor, SK&F 105657: role for testosterone in the androgenic response." *Endocrinology* **130**(2): 685-94.
- Lambright, C., J. Ostby, K. Bobseine, V. Wilson, A. K. Hotchkiss, P. C. Mann and L. E. Gray, Jr. (2000). "Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats." *Toxicol Sci* **56**(2): 389-99.

- Lang, S. H., R. M. Sharrard, M. Stark, J. M. Villette and N. J. Maitland (2001). "Prostate epithelial cell lines form spheroids with evidence of glandular differentiation in three-dimensional Matrigel cultures." *Br J Cancer* **85**(4): 590-9.
- Lee, E. Y., G. Parry and M. J. Bissell (1984). "Modulation of secreted proteins of mouse mammary epithelial cells by the collagenous substrata." *J Cell Biol* **98**(1): 146-55.
- Lee, H. and D. Kimelman (2002). "A dominant-negative form of p63 is required for epidermal proliferation in zebrafish." *Dev Cell* **2**(5): 607-16.
- Lehmann, K. P., S. Phillips, M. Sar, P. M. Foster and K. W. Gaido (2004). "Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate." *Toxicol Sci* **81**(1): 60-8.
- Lejeune, B., J. Van Hoesck and F. Leroy (1981). "Transmitter role of the luminal uterine epithelium in the induction of decidualization in rats." *J Reprod Fertil* **61**(1): 235-40.
- Levine, M. and T. Hoey (1988). "Homeobox proteins as sequence-specific transcription factors." *Cell* **55**(4): 537-40.
- Levrero, M., V. De Laurenzi, A. Costanzo, J. Gong, J. Y. Wang and G. Melino (2000). "The p53/p63/p73 family of transcription factors: overlapping and distinct functions." *J Cell Sci* **113** (Pt 10): 1661-70.
- Lim, H. N. and J. R. Hawkins (1998). "Genetic control of gonadal differentiation." *Baillieres Clin Endocrinol Metab* **12**(1): 1-16.
- Lin, H. K., S. Altuwaijri, W. J. Lin, P. Y. Kan, L. L. Collins and C. Chang (2002). "Proteasome activity is required for androgen receptor transcriptional activity via regulation of androgen receptor nuclear translocation and interaction with coregulators in prostate cancer cells." *J Biol Chem* **277**(39): 36570-6.
- Lindqvist, A., I. A. Hughes and S. Andersson (2001). "Substitution mutation C268Y causes 17 beta-hydroxysteroid dehydrogenase 3 deficiency." *J Clin Endocrinol Metab* **86**(2): 921-3.
- Lovell-Badge, R. and E. Robertson (1990). "XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy." *Development* **109**(3): 635-46.
- Lubahn, D. B., T. R. Brown, J. A. Simental, H. N. Higgs, C. J. Migeon, E. M. Wilson and F. S. French (1989). "Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity." *Proc Natl Acad Sci U S A* **86**(23): 9534-8.
- Lubahn, D. B., J. S. Moyer, T. S. Golding, J. F. Couse, K. S. Korach and O. Smithies (1993). "Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene." *Proc Natl Acad Sci U S A* **90**(23): 11162-6.
- Lyon, M. F. and S. G. Hawkes (1970). "X-linked gene for testicular feminization in the mouse." *Nature* **227**(5264): 1217-9.
- MacLaughlin, D. T. and P. K. Donahoe (2004). "Sex determination and differentiation." *N Engl J Med* **350**(4): 367-78.
- MacLaughlin, D. T., J. Teixeira and P. K. Donahoe (2001). "Perspective: reproductive tract development--new discoveries and future directions." *Endocrinology* **142**(6): 2167-72.
- Magro, G. and S. Grasso (1995). "Expression of cytokeratins, vimentin and basement membrane components in human fetal male mullerian duct and perimullerian mesenchyme." *Acta Histochem* **97**(1): 13-8.
- Mahood, I. K., N. Hallmark, C. McKinnell, M. Walker, J. S. Fisher and R. M. Sharpe (2005). "Abnormal Leydig Cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis." *Endocrinology* **146**(2): 613-23.
- Majdic, G., M. R. Millar and P. T. Saunders (1995). "Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts." *J Endocrinol* **147**(2): 285-93.
- Majdic, G., P. T. Saunders and K. J. Teerds (1998). "Immunoexpression of the steroidogenic enzymes 3-beta hydroxysteroid dehydrogenase and 17 alpha-hydroxylase, C17,20 lyase and the receptor for luteinizing hormone (LH) in the fetal rat testis suggests that the onset of Leydig cell steroid production is independent of LH action." *Biol Reprod* **58**(2): 520-5.
- Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon and R. M. Evans (1995). "The nuclear receptor superfamily: the second decade." *Cell* **83**(6): 835-9.

- Manikkam, M., E. J. Crespi, D. D. Doop, C. Herkimer, J. S. Lee, S. Yu, M. B. Brown, D. L. Foster and V. Padmanabhan (2004). "Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep." *Endocrinology* **145**(2): 790-8.
- Marcelli, M., S. Zoppi, P. B. Grino, J. E. Griffin, J. D. Wilson and M. J. McPhaul (1991). "A mutation in the DNA-binding domain of the androgen receptor gene causes complete testicular feminization in a patient with receptor-positive androgen resistance." *J Clin Invest* **87**(3): 1123-6.
- Marker, P. C., A. A. Donjacour, R. Dahiya and G. R. Cunha (2003). "Hormonal, cellular, and molecular control of prostatic development." *Dev Biol* **253**(2): 165-74.
- McElreavey, K., E. Vilain, N. Abbas, I. Herskowitz and M. Fellous (1993). "A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development." *Proc Natl Acad Sci U S A* **90**(8): 3368-72.
- McEwan, I. J. (2004). "Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain." *Endocr Relat Cancer* **11**(2): 281-93.
- McEwan, I. J. (2004). "Sex, drugs and gene expression: signalling by members of the nuclear receptor superfamily." *Essays Biochem* **40**: 1-10.
- McIntyre, B. S., N. J. Barlow and P. M. Foster (2001). "Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues." *Toxicol Sci* **62**(2): 236-49.
- McIntyre, B. S., N. J. Barlow, M. Sar, D. G. Wallace and P. M. Foster (2002). "Effects of in utero linuron exposure on rat Wolffian duct development." *Reprod Toxicol* **16**(2): 131-9.
- McIntyre, B. S., N. J. Barlow, D. G. Wallace, S. C. Maness, K. W. Gaido and P. M. Foster (2000). "Effects of in utero exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat." *Toxicol Appl Pharmacol* **167**(2): 87-99.
- McKenna, N. J., R. B. Lanz and B. W. O'Malley (1999). "Nuclear receptor coregulators: cellular and molecular biology." *Endocr Rev* **20**(3): 321-44.
- McKinnell, C., R. M. Sharpe, K. Mahood, N. Hallmark, H. Scott, R. Ivell, C. Staub, B. Jegou, F. Haag, F. Koch-Nolte and S. Hartung (2005). "Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by in utero exposure to di (n-Butyl) phthalate." *Endocrinology* **146**(10): 4536-44.
- McLeod, D. G., R. C. Benson, Jr., M. A. Eisenberger, E. D. Crawford, B. A. Blumenstein, D. Spicer and J. T. Spaulding (1993). "The use of flutamide in hormone-refractory metastatic prostate cancer." *Cancer* **72**(12 Suppl): 3870-3.
- McPhaul, M. J. (2002). "Androgen receptor mutations and androgen insensitivity." *Mol Cell Endocrinol* **198**(1-2): 61-7.
- Meaney, M. J. and J. Stewart (1981). "Neonatal-androgens influence the social play of prepubescent rats." *Horm Behav* **15**(2): 197-213.
- Menke, D. B. and D. C. Page (2002). "Sexually dimorphic gene expression in the developing mouse gonad." *Gene Expr Patterns* **2**(3-4): 359-67.
- Merke, D. P. and S. R. Bornstein (2005). "Congenital adrenal hyperplasia." *Lancet* **365**(9477): 2125-36.
- Migliaccio, A., G. Castoria, M. Di Domenico, A. de Falco, A. Bilancio, M. Lombardi, M. V. Barone, D. Ametrano, M. S. Zannini, C. Abbondanza and F. Auricchio (2000). "Steroid-induced androgen receptor-oestradiol receptor beta- β -Src complex triggers prostate cancer cell proliferation." *Embo J* **19**(20): 5406-17.
- Migliaccio, A., M. Di Domenico, S. Green, A. de Falco, E. L. Kajtaniak, F. Blasi, P. Chambon and F. Auricchio (1989). "Phosphorylation on tyrosine of in vitro synthesized human estrogen receptor activates its hormone binding." *Mol Endocrinol* **3**(7): 1061-9.
- Milewich, L., F. W. George and J. D. Wilson (1977). "Estrogen formation by the ovary of the rabbit embryo." *Endocrinology* **100**(1): 187-96.
- Miller, W. L. (1998). "Early steps in androgen biosynthesis: from cholesterol to DHEA." *Baillieres Clin Endocrinol Metab* **12**(1): 67-81.
- Mittwoch, U. (1969). "Do genes determine sex?" *Nature* **221**(179): 446-8.
- Morris, J. M. (1953). "The syndrome of testicular feminization in male pseudohermaphrodites." *Am J Obstet Gynecol* **65**(6): 1192-1211.
- Munsterberg, A. and R. Lovell-Badge (1991). "Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation." *Development* **113**(2): 613-24.

- Murphy, L. and P. J. O'Shaughnessy (1991). "Testicular steroidogenesis in the testicular feminized (Tfm) mouse: loss of 17 alpha-hydroxylase activity." *J Endocrinol* **131**(3): 443-449.
- Mylchreest, E., R. C. Cattley and P. M. Foster (1998). "Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism?" *Toxicol Sci* **43**(1): 47-60.
- Mylchreest, E. and P. M. Foster (2000). "DBP exerts its antiandrogenic activity by indirectly interfering with androgen signaling pathways." *Toxicol Appl Pharmacol* **168**(2): 174-5.
- Mylchreest, E., M. Sar, R. C. Cattley and P. M. Foster (1999). "Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide." *Toxicol Appl Pharmacol* **156**(2): 81-95.
- Mylchreest, E., D. G. Wallace, R. C. Cattley and P. M. Foster (2000). "Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyl) phthalate during late gestation." *Toxicol Sci* **55**(1): 143-51.
- Nachtigal, M. W., Y. Hirokawa, D. L. Enyeart-VanHouten, J. N. Flanagan, G. D. Hammer and H. A. Ingraham (1998). "Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression." *Cell* **93**(3): 445-54.
- Nef, S. and L. F. Parada (1999). "Cryptorchidism in mice mutant for Ins13." *Nat Genet* **22**(3): 295-9.
- Nef, S. and L. F. Parada (2000). "Hormones in male sexual development." *Genes Dev* **14**(24): 3075-86.
- Nef, S., T. Shipman and L. F. Parada (2000). "A molecular basis for estrogen-induced cryptorchidism." *Dev Biol* **224**(2): 354-61.
- Neri, R. O. and M. Monahan (1972). "Effects of a novel nonsteroidal antiandrogen on canine prostatic hyperplasia." *Invest Urol* **10**(2): 123-30.
- Newbold, R. R. and J. A. McLachlan (1982). "Vaginal adenosis and adenocarcinoma in mice exposed prenatally or neonatally to diethylstilbestrol." *Cancer Res* **42**(5): 2003-11.
- O'Rahilly, R. (1977). "The development of the vagina in the human." *Birth Defects Orig Artic Ser* **13**(2): 123-36.
- O'Shaughnessy, P. J., P. Baker, U. Sohnius, A. M. Haavisto, H. M. Charlton and I. Huhtaniemi (1998). "Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function." *Endocrinology* **139**(3): 1141-6.
- O'Shaughnessy, P. J., P. J. Baker and H. Johnston (2005). "Neuroendocrine regulation of Leydig cell development." *Ann N Y Acad Sci* **1061**: 109-19.
- O'Shaughnessy, P. J. and L. Murphy (1993). "Cytochrome P-450 17 alpha-hydroxylase protein and mRNA in the testis of the testicular feminized (Tfm) mouse." *J Mol Endocrinol* **11**(1): 77-82.
- Ogawa, S. and A. Nozawa (1969). "Effect of prenatal administration of androgen on the reproductive function in female rats." *Endocrinol Jpn* **16**(6): 599-607.
- Onate, S. A., S. Y. Tsai, M. J. Tsai and B. W. O'Malley (1995). "Sequence and characterization of a coactivator for the steroid hormone receptor superfamily." *Science* **270**(5240): 1354-7.
- Orgebin-Crist, M. C., B. C. Eller and B. J. Danzo (1983). "The effects of estradiol, tamoxifen, and testosterone on the weights and histology of the epididymis and accessory sex organs of sexually immature rabbits." *Endocrinology* **113**(5): 1703-15.
- Orti, E., J. E. Bodwell and A. Munck (1992). "Phosphorylation of steroid hormone receptors." *Endocr Rev* **13**(1): 105-28.
- Orti, E., D. B. Mendel, L. I. Smith and A. Munck (1989). "Agonist-dependent phosphorylation and nuclear dephosphorylation of glucocorticoid receptors in intact cells." *J Biol Chem* **264**(17): 9728-31.
- Padmanabhan, V., Evans, N., Taylor, J.A., and Robinson, J.E. (1998). "Prenatal exposure to androgens leads to the development of cystic ovaries in the sheep." *Biology of Reproduction* **56** (Suppl 1) 194.
- Padmanabhan, V., M. Manikkam, S. Recabarren and D. Foster (2006). "Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female." *Mol Cell Endocrinol* **246**(1-2): 165-74.
- Paramio, J. M. and J. L. Jorcano (2002). "Beyond structure: do intermediate filaments modulate cell signalling?" *Bioessays* **24**(9): 836-44.
- Paranko, J., L. J. Pelliniemi and J. M. Foidart (1984). "Epithelio-mesenchymal interface and fibronectin in the differentiation of the rat mesonephric and paramesonephric ducts." *Differentiation* **27**(3): 196-204.

- Paranko, J., L. J. Pelliniemi, A. Vaheri, J. M. Foidart and T. Lakkala-Paranko (1983). "Morphogenesis and fibronectin in sexual differentiation of rat embryonic gonads." *Differentiation* **23 Suppl**: S72-81.
- Park, S. Y., J. J. Meeks, G. Raverot, L. E. Pfaff, J. Weiss, G. D. Hammer and J. L. Jameson (2005). "Nuclear receptors Sfl and Dax1 function cooperatively to mediate somatic cell differentiation during testis development." *Development* **132**(10): 2415-23.
- Parker, M. G. (1990). "Structure and function of nuclear hormone receptors." *Semin Cancer Biol* **1**(1): 81-7.
- Parks, L. G., J. S. Ostby, C. R. Lambright, B. D. Abbott, G. R. Klinefelter, N. J. Barlow and L. E. Gray, Jr. (2000). "The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat." *Toxicol Sci* **58**(2): 339-49.
- Patterson, M. N., M. J. McPhaul and I. A. Hughes (1994). "Androgen insensitivity syndrome." *Baillieres Clin Endocrinol Metab* **8**(2): 379-404.
- Peets, E. A., M. F. Henson and R. Neri (1974). "On the mechanism of the anti-androgenic action of flutamide (alpha-alpha-alpha-trifluoro-2-methyl-4'-nitro-m-propionotoluidide) in the rat." *Endocrinology* **94**(2): 532-40.
- Pelletier, G., C. Labrie and F. Labrie (2000). "Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs." *J Endocrinol* **165**(2): 359-70.
- Picard, D., B. Khursheed, M. J. Garabedian, M. G. Fortin, S. Lindquist and K. R. Yamamoto (1990). "Reduced levels of hsp90 compromise steroid receptor action in vivo." *Nature* **348**(6297): 166-8.
- Planz, B., H. T. Aretz, Q. Wang, S. Tabatabaei, S. D. Kirley, C. W. Lin and W. S. McDougal (1999). "Immunolocalization of the keratinocyte growth factor in benign and neoplastic human prostate and its relation to androgen receptor." *Prostate* **41**(4): 233-42.
- Podlasek, C. A., R. M. Seo, J. Q. Clemens, L. Ma, R. L. Maas and W. Bushman (1999). "Hoxa-10 deficient male mice exhibit abnormal development of the accessory sex organs." *Dev Dyn* **214**(1): 1-12.
- Pointis, G., M. T. Latreille and L. Cedard (1980). "Gonado-pituitary relationships in the fetal mouse at various times during sexual differentiation." *J Endocrinol* **86**(3): 483-8.
- Price, D., and Oritiz, E. (1965). The role of fetal androgens in sex differentiation in mammals. In: *Organogenesis*. R. L. Ursprung, and DeHaan, H. New York, Holt, Rinehart, and Winston: 629-652.
- Price, J. M., P. K. Donahoe, Y. Ito and W. H. Hendren, 3rd (1977). "Programmed cell death in the Mullerian duct induced by Mullerian inhibiting substance." *Am J Anat* **149**(3): 353-75.
- Prins, G. S. and L. Birch (1993). "Immunocytochemical analysis of androgen receptor along the ducts of the separate rat prostate lobes after androgen withdrawal and replacement." *Endocrinology* **132**(1): 169-78.
- Prins, G. S., L. Birch, J. F. Couse, I. Choi, B. Katzenellenbogen and K. S. Korach (2001). "Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor alpha: studies with alphaERKO and betaERKO mice." *Cancer Res* **61**(16): 6089-97.
- Qin, Y. and C. E. Bishop (2005). "Sox9 is sufficient for functional testis development producing fertile male mice in the absence of Sry." *Hum Mol Genet* **14**(9): 1221-9.
- Quigley, C. A. (2002). "Editorial: The postnatal gonadotropin and sex steroid surge-insights from the androgen insensitivity syndrome." *J Clin Endocrinol Metab* **87**(1): 24-8.
- Quigley, C. A., A. De Bellis, K. B. Marschke, M. K. el-Awady, E. M. Wilson and F. S. French (1995). "Androgen receptor defects: historical, clinical, and molecular perspectives." *Endocr Rev* **16**(3): 271-321.
- Quigley, C. A., B. A. Evans, J. A. Simental, K. B. Marschke, M. Sar, D. B. Lubahn, P. Davies, I. A. Hughes, E. M. Wilson and F. S. French (1992). "Complete androgen insensitivity due to deletion of exon C of the androgen receptor gene highlights the functional importance of the second zinc finger of the androgen receptor in vivo." *Mol Endocrinol* **6**(7): 1103-12.
- Rey, R. and J. Y. Picard (1998). "Embryology and endocrinology of genital development." *Baillieres Clin Endocrinol Metab* **12**(1): 17-33.
- Reyes, F. I., J. S. Winter and C. Faiman (1973). "Studies on human sexual development. I. Fetal gonadal and adrenal sex steroids." *J Clin Endocrinol Metab* **37**(1): 74-8.

- Ris-Stalpers, C., G. G. Kuiper, P. W. Faber, H. U. Schweikert, H. C. van Rooij, N. D. Zegers, M. B. Hodgins, H. J. Degenhart, J. Trapman and A. O. Brinkmann (1990). "Aberrant splicing of androgen receptor mRNA results in synthesis of a nonfunctional receptor protein in a patient with androgen insensitivity." *Proc Natl Acad Sci U S A* **87**(20): 7866-70.
- Risbridger, G., H. Wang, P. Young, T. Kurita, Y. Z. Wang, D. Lubahn, J. A. Gustafsson and G. Cunha (2001). "Evidence that epithelial and mesenchymal estrogen receptor- α mediates effects of estrogen on prostatic epithelium." *Dev Biol* **229**(2): 432-42.
- Risbridger, G. P., H. Wang, M. Frydenberg and G. Cunha (2001). "The metaplastic effects of estrogen on mouse prostate epithelium: proliferation of cells with basal cell phenotype." *Endocrinology* **142**(6): 2443-50.
- Robaire, B. and N. A. Henderson (2006). "Actions of 5 α -reductase inhibitors on the epididymis." *Mol Cell Endocrinol* **250**(1-2): 190-5.
- Robaire, B. a. H., L. (1988). *Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation*. In: *Physiology of Reproduction*. New York, Raven Press.
- Roberts, L. M., Y. Hirokawa, M. W. Nachtigal and H. A. Ingraham (1999). "Paracrine-mediated apoptosis in reproductive tract development." *Dev Biol* **208**(1): 110-22.
- Roberts, L. M., J. A. Visser and H. A. Ingraham (2002). "Involvement of a matrix metalloproteinase in MIS-induced cell death during urogenital development." *Development* **129**(6): 1487-96.
- Robertson, K. M., L. O'Donnell, M. E. Jones, S. J. Meachem, W. C. Boon, C. R. Fisher, K. H. Graves, R. I. McLachlan and E. R. Simpson (1999). "Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene." *Proc Natl Acad Sci U S A* **96**(14): 7986-91.
- Roche, P. J., S. A. Hoare and M. G. Parker (1992). "A consensus DNA-binding site for the androgen receptor." *Mol Endocrinol* **6**(12): 2229-35.
- Rosenfeld, M. G. and C. K. Glass (2001). "Coregulator codes of transcriptional regulation by nuclear receptors." *J Biol Chem* **276**(40): 36865-8.
- Rowlands, T. M., J. M. Symonds, R. Farookhi and O. W. Blaschuk (2000). "Cadherins: crucial regulators of structure and function in reproductive tissues." *Rev Reprod* **5**(1): 53-61.
- Ruoslahti, E. (1981). "Fibronectin." *J Oral Pathol* **10**(1): 3-13.
- Russell, D. W. and J. D. Wilson (1994). "Steroid 5 α -reductase: two genes/two enzymes." *Annu Rev Biochem* **63**: 25-61.
- Saitoh, M., R. Takayanagi, K. Goto, A. Fukamizu, A. Tomura, T. Yanase and H. Nawata (2002). "The presence of both the amino- and carboxyl-terminal domains in the AR is essential for the completion of a transcriptionally active form with coactivators and intranuclear compartmentalization common to the steroid hormone receptors: a three-dimensional imaging study." *Mol Endocrinol* **16**(4): 694-706.
- Sajjad, Y., S. Quenby, P. Nickson, D. I. Lewis-Jones and G. Vince (2004). "Immunohistochemical localization of androgen receptors in the urogenital tracts of human embryos." *Reproduction* **128**(3): 331-9.
- Sakakura, T., Y. Sakagami and Y. Nishizuka (1979). "Persistence of responsiveness of adult mouse mammary gland to induction by embryonic mesenchyme." *Dev Biol* **72**(2): 201-10.
- Sassoon, D. (1999). "Wnt genes and endocrine disruption of the female reproductive tract: a genetic approach." *Mol Cell Endocrinol* **158**(1-2): 1-5.
- Saunders, P. T. (1998). "Oestrogen receptor beta (ER beta)." *Rev Reprod* **3**(3): 164-71.
- Saunders, P. T., J. S. Fisher, R. M. Sharpe and M. R. Millar (1998). "Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis." *J Endocrinol* **156**(3): R13-7.
- Saunders, P. T., T. Padayachi, D. G. Tincello, S. M. Shalet and F. C. Wu (1992). "Point mutations detected in the androgen receptor gene of three men with partial androgen insensitivity syndrome." *Clin Endocrinol (Oxf)* **37**(3): 214-20.
- Schaufele, F., X. Carbonell, M. Guerbodot, S. Borngraeber, M. S. Chapman, A. A. Ma, J. N. Miner and M. I. Diamond (2005). "The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions." *Proc Natl Acad Sci U S A* **102**(28): 9802-7.
- Schedin, P., R. Strange, T. Mitrenga, P. Wolfe and M. Kaeck (2000). "Fibronectin fragments induce MMP activity in mouse mammary epithelial cells: evidence for a role in mammary tissue remodeling." *J Cell Sci* **113** (Pt 5): 795-806.
- Schlatt, S., G. F. Weinbauer, M. Arslan and E. Nieschlag (1993). "Appearance of alpha-smooth muscle actin in peritubular cells of monkey testes is induced by androgens, modulated by

- follicle-stimulating hormone, and maintained after hormonal withdrawal." *J Androl* **14**(5): 340-50.
- Schoenmakers, E., P. Alen, G. Verrijdt, B. Peeters, G. Verhoeven, W. Rombauts and F. Claessens (1999). "Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains." *Biochem J* **341** (Pt 3): 515-21.
- Seckl, J. R. (2004). "Prenatal glucocorticoids and long-term programming." *Eur J Endocrinol* **151** Suppl 3: U49-62.
- Sharpe, R. M. (1990). "Intratesticular control of steroidogenesis." *Clin Endocrinol (Oxf)* **33**(6): 787-807.
- Sharpe, R. M. (1998). "The roles of oestrogen in the male." *Trends Endocrinol. Metab.* **9**: 371-377.
- Shi, S. R., B. Chaiwun, L. Young, R. J. Cote and C. R. Taylor (1993). "Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections." *J Histochem Cytochem* **41**(11): 1599-604.
- Shook, D. and R. Keller (2003). "Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development." *Mech Dev* **120**(11): 1351-83.
- Siiteri, P. K. and J. D. Wilson (1974). "Testosterone formation and metabolism during male sexual differentiation in the human embryo." *J Clin Endocrinol Metab* **38**(1): 113-25.
- Silversides, D. W., C. A. Price and G. M. Cooke (1995). "Effects of short-term exposure to hydroxyflutamide in utero on the development of the reproductive tract in male mice." *Can J Physiol Pharmacol* **73**(11): 1582-8.
- Simard, J., I. Luthy, J. Guay, A. Belanger and F. Labrie (1986). "Characteristics of interaction of the antiandrogen flutamide with the androgen receptor in various target tissues." *Mol Cell Endocrinol* **44**(3): 261-70.
- Simental, J. A., M. Sar, M. V. Lane, F. S. French and E. M. Wilson (1991). "Transcriptional activation and nuclear targeting signals of the human androgen receptor." *J Biol Chem* **266**(1): 510-8.
- Singh, S., S. Sadacharan, S. Su, A. Beldegrun, S. Persad and G. Singh (2003). "Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer." *Cancer Res* **63**(9): 2306-11.
- Sir-Petermann, T. (2005). "Polycystic ovary syndrome, a pathway to type 2 diabetes." *Nutrition* **21**(11-12): 1160-3.
- Sir-Petermann, T., C. Hittsfield, M. Maliqueo, E. Codner, B. Echiburu, R. Gazitua, S. Recabarren and F. Cassorla (2005). "Birth weight in offspring of mothers with polycystic ovarian syndrome." *Hum Reprod* **20**(8): 2122-6.
- Staaack, A., A. A. Donjacour, J. Brody, G. R. Cunha and P. Carroll (2003). "Mouse urogenital development: a practical approach." *Differentiation* **71**(7): 402-13.
- Steckler, T., J. Wang, F. F. Bartol, S. K. Roy and V. Padmanabhan (2005). "Fetal programming: prenatal testosterone treatment causes intrauterine growth retardation, reduces ovarian reserve and increases ovarian follicular recruitment." *Endocrinology* **146**(7): 3185-93.
- Stinnakre, M. G. (1975). "[Period of sensitivity to androgens of the Wolff duct of the rat fetus]." *Arch Anat Microsc Morphol Exp* **64**(1): 45-59.
- Stocco, D. M. and B. J. Clark (1996). "Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis." *Biochem Pharmacol* **51**(3): 197-205.
- Streuli, C. H. and A. P. Gilmore (1999). "Adhesion-mediated signaling in the regulation of mammary epithelial cell survival." *J Mammary Gland Biol Neoplasia* **4**(2): 183-91.
- Stumpf, W. E., R. Narbaitz and M. Sar (1980). "Estrogen receptors in the fetal mouse." *J Steroid Biochem* **12**: 55-64.
- Sugimura, Y., G. R. Cunha and A. A. Donjacour (1986). "Morphological and histological study of castration-induced degeneration and androgen-induced regeneration in the mouse prostate." *Biol Reprod* **34**(5): 973-83.
- Sugimura, Y., B. A. Foster, Y. K. Hom, J. H. Lipschutz, J. S. Rubin, P. W. Finch, S. A. Aaronson, N. Hayashi, J. Kawamura and G. R. Cunha (1996). "Keratinocyte growth factor (KGF) can replace testosterone in the ductal branching morphogenesis of the rat ventral prostate." *Int J Dev Biol* **40**(5): 941-51.
- Swain, A. and R. Lovell-Badge (1999). "Mammalian sex determination: a molecular drama." *Genes Dev* **13**(7): 755-67.
- Swain, A., V. Narvaez, P. Burgoyne, G. Camerino and R. Lovell-Badge (1998). "Dax1 antagonizes Sry action in mammalian sex determination." *Nature* **391**(6669): 761-7.

- Swan, S. H., K. M. Main, F. Liu, S. L. Stewart, R. L. Kruse, A. M. Calafat, C. S. Mao, J. B. Redmon, C. L. Ternand, S. Sullivan and J. L. Teague (2005). "Decrease in anogenital distance among male infants with prenatal phthalate exposure." *Environ Health Perspect* **113**(8): 1056-61.
- Swanson, H. E. and J. J. Werff ten Bosch (1965). "The "early-androgen" syndrome; effects of pre-natal testosterone propionate." *Acta Endocrinol (Copenh)* **50**(3): 379-90.
- Swerdloff, R. S. a. H., D. (1981). *Endocrine Control of Testicular Function From Birth to Puberty*. New York, Raven Press.
- Tan, J. A., D. R. Joseph, V. E. Quarmby, D. B. Lubahn, M. Sar, F. S. French and E. M. Wilson (1988). "The rat androgen receptor: primary structure, autoregulation of its messenger ribonucleic acid, and immunocytochemical localization of the receptor protein." *Mol Endocrinol* **2**(12): 1276-85.
- Taneja, S. S., S. Ha, N. K. Swenson, H. Y. Huang, P. Lee, J. Melamed, E. Shapiro, M. J. Garabedian and S. K. Logan (2005). "Cell-specific regulation of androgen receptor phosphorylation in vivo." *J Biol Chem* **280**(49): 40916-24.
- Tapanainen, J., P. Kellokumpu-Lehtinen, L. Pelliniemi and I. Huhtaniemi (1981). "Age-related changes in endogenous steroids of human fetal testis during early and midpregnancy." *J Clin Endocrinol Metab* **52**(1): 98-102.
- Tavella, S., G. Bellese, P. Castagnola, I. Martin, D. Piccini, R. Doliana, A. Colombatti, R. Cancedda and C. Tacchetti (1997). "Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation." *J Cell Sci* **110** (Pt 18): 2261-70.
- Taylor, H. S., A. Arici, D. Olive and P. Igarashi (1998). "HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium." *J Clin Invest* **101**(7): 1379-84.
- Taylor, H. S., C. Bagot, A. Kardana, D. Olive and A. Arici (1999). "HOX gene expression is altered in the endometrium of women with endometriosis." *Hum Reprod* **14**(5): 1328-31.
- Taylor, H. S., P. Igarashi, D. L. Olive and A. Arici (1999). "Sex steroids mediate HOXA11 expression in the human peri-implantation endometrium." *J Clin Endocrinol Metab* **84**(3): 1129-35.
- Teixeira, J. and P. K. Donahoe (1996). "Molecular biology of MIS and its receptors." *J Androl* **17**(4): 336-41.
- Themmen, A. P. N. and I. T. Huhtaniemi (2000). "Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function." *Endocr Rev* **21**(5): 551-83.
- Thiery, J. P. (2002). "Epithelial-mesenchymal transitions in tumour progression." *Nat Rev Cancer* **2**(6): 442-54.
- Thigpen, A. E., R. I. Silver, J. M. Guileyardo, M. L. Casey, J. D. McConnell and D. W. Russell (1993). "Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression." *J Clin Invest* **92**(2): 903-10.
- Thomson, A. A. and P. C. Marker (2006). "Branching morphogenesis in the prostate gland and seminal vesicles." *Differentiation* **74**(7): 382-92.
- Thomson, A. A., B. G. Timms, L. Barton, G. R. Cunha and O. C. Grace (2002). "The role of smooth muscle in regulating prostatic induction." *Development* **129**(8): 1905-12.
- Timms, B. G., C. W. Lee, G. Aumuller and J. Seitz (1995). "Instructive induction of prostate growth and differentiation by a defined urogenital sinus mesenchyme." *Microsc Res Tech* **30**(4): 319-32.
- Tomsig, J. L. and T. T. Turner (2006). "Growth factors and the epididymis." *J Androl* **27**(3): 348-57.
- Tomsig, J. L., S. Usanovic and T. T. Turner (2006). "Growth Factor-Stimulated Mitogen Activated Kinase (MAPK) Phosphorylation in the Rat Epididymis is Limited by Segmental Boundaries." *Biol Reprod*.
- Tong, S. Y., J. M. Hutson and L. M. Watts (1996). "Does testosterone diffuse down the wolffian duct during sexual differentiation?" *J Urol* **155**(6): 2057-9.
- Traish, A. M. and H. H. Wotiz (1987). "Prostatic epidermal growth factor receptors and their regulation by androgens." *Endocrinology* **121**(4): 1461-7.
- Trelstad, R. L., A. Hayashi, K. Hayashi and P. K. Donahoe (1982). "The epithelial-mesenchymal interface of the male rat Mullerian duct: loss of basement membrane integrity and ductal regression." *Dev Biol* **92**(1): 27-40.
- Tsuji, M., H. Shima and G. R. Cunha (1991). "In vitro androgen-induced growth and morphogenesis of the Wolffian duct within urogenital ridge." *Endocrinology* **128**(4): 1805-11.

- Tsuji, M., H. Shima, N. Terada and G. R. Cunha (1994). "5 alpha-reductase activity in developing urogenital tracts of fetal and neonatal male mice." *Endocrinology* **134**(5): 2198-205.
- Tsuji, M., H. Shima, C. Y. Yonemura, J. Brody, P. K. Donahoe and G. R. Cunha (1992). "Effect of human recombinant mullerian inhibiting substance on isolated epithelial and mesenchymal cells during mullerian duct regression in the rat." *Endocrinology* **131**(3): 1481-8.
- Turner, K. J., B. S. McIntyre, S. L. Phillips, N. J. Barlow, C. J. Bowman and P. M. Foster (2003). "Altered gene expression during rat Wolffian duct development in response to in utero exposure to the antiandrogen linuron." *Toxicol Sci* **74**(1): 114-28.
- Tut, T. G., F. J. Ghadessy, M. A. Trifiro, L. Pinsky and E. L. Yong (1997). "Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility." *J Clin Endocrinol Metab* **82**(11): 3777-82.
- Vainio, S., M. Heikkila, A. Kispert, N. Chin and A. P. McMahon (1999). "Female development in mammals is regulated by Wnt-4 signalling." *Nature* **397**(6718): 405-9.
- Van Doorn, E. and N. Bruchofsky (1978). "Mechanisms of replenishment of nuclear androgen receptor in rat ventral prostate." *Biochem J* **174**(1): 9-16.
- van Laar, J. H., C. A. Berrevoets, J. Trapman, N. D. Zegers and A. O. Brinkmann (1991). "Hormone-dependent androgen receptor phosphorylation is accompanied by receptor transformation in human lymph node carcinoma of the prostate cells." *J Biol Chem* **266**(6): 3734-8.
- van Laar, J. H., J. Bolt-de Vries, N. D. Zegers, J. Trapman and A. O. Brinkmann (1990). "Androgen receptor heterogeneity and phosphorylation in human LNCaP cells." *Biochem Biophys Res Commun* **166**(1): 193-200.
- Verhoeven, G. and J. V. Swinnen (1999). "Indirect mechanisms and cascades of androgen action." *Mol Cell Endocrinol* **151**(1-2): 205-12.
- Veyssiere, G., M. Berger, C. Jean-Faucher, M. de Turckheim and C. Jean (1982). "Testosterone and dihydrotestosterone in sexual ducts and genital tubercle of rabbit fetuses during sexual organogenesis: effects of fetal decapitation." *J Steroid Biochem* **17**(2): 149-54.
- Waller, C. L., B. W. Juma, L. E. Gray, Jr. and W. R. Kelce (1996). "Three-dimensional quantitative structure-activity relationships for androgen receptor ligands." *Toxicol Appl Pharmacol* **137**(2): 219-27.
- Wang, G. M., P. J. O'Shaughnessy, C. Chubb, B. Robaire and M. P. Hardy (2003). "Effects of insulin-like growth factor I on steroidogenic enzyme expression levels in mouse leydig cells." *Endocrinology* **144**(11): 5058-64.
- Wang, M., J. Wang, Z. Zhang, Z. Zhao, R. Zhang, X. Hu, T. Tan, S. Luo and Z. Luo (2005). "Dissecting phenotypic variation among AIS patients." *Biochem Biophys Res Commun* **335**(2): 335-42.
- Wang, T. Y., B. F. Chen, Y. C. Yang, H. Chen, Y. Wang, A. Cviko, B. J. Quade, D. Sun, A. Yang, F. D. McKeon and C. P. Crum (2001). "Histologic and immunophenotypic classification of cervical carcinomas by expression of the p53 homologue p63: a study of 250 cases." *Hum Pathol* **32**(5): 479-86.
- Warren, D. W., G. C. Haltmeyer and K. B. Eik-Nes (1972). "Synthesis and metabolism of testosterone in the fetal rat testis." *Biol Reprod* **7**(1): 94-9.
- Warren, D. W., G. C. Haltmeyer and K. B. Eik-Nes (1975). "The effect of gonadotrophins on the fetal and neonatal rat testis." *Endocrinology* **96**(5): 1226-9.
- Weisz, J. and I. L. Ward (1980). "Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring." *Endocrinology* **106**(1): 306-16.
- Welshons, W. J. and L. B. Russell (1959). "The Y-Chromosome as the Bearer of Male Determining Factors in the Mouse." *Proc Natl Acad Sci U S A* **45**(4): 560-6.
- Williams, K., J. S. Fisher, K. J. Turner, C. McKinnell, P. T. Saunders and R. M. Sharpe (2001). "Relationship between expression of sex steroid receptors and structure of the seminal vesicles after neonatal treatment of rats with potent or weak estrogens." *Environ Health Perspect* **109**(12): 1227-35.
- Williams, K., C. McKinnell, P. T. Saunders, M. Walker, J. S. Fisher, K. J. Turner, N. Atanassova and M. Sharpe (2001). "Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man." *Hum Reprod Update* **7**(3): 236-47.
- Wilson, C. M. and M. J. McPhaul (1996). "A and B forms of the androgen receptor are expressed in a variety of human tissues." *Mol Cell Endocrinol* **120**(1): 51-7.

- Wilson, J. D. (1996). "Role of dihydrotestosterone in androgen action." *Prostate Suppl* **6**: 88-92.
- Wilson, J. D., F. W. George and J. E. Griffin (1981). "The hormonal control of sexual development." *Science* **211**(4488): 1278-84.
- Wilson, J. D., J. E. Griffin, F. W. George and M. Leshin (1981). "The role of gonadal steroids in sexual differentiation." *Recent Prog Horm Res* **37**: 1-39.
- Wilson, J. D., M. J. Harrod, J. L. Goldstein, D. L. Hemsell and P. C. MacDonald (1974). "Familial incomplete male pseudohermaphroditism, type 1. Evidence for androgen resistance and variable clinical manifestations in a family with the Reifenstein syndrome." *N Engl J Med* **290**(20): 1097-103.
- Wilson, J. D. and I. Lasnitzki (1971). "Dihydrotestosterone formation in fetal tissues of the rabbit and rat." *Endocrinology* **89**(3): 659-68.
- Wilson, V. S., C. Lambricht, J. Furr, J. Ostby, C. Wood, G. Held and L. E. Gray, Jr. (2004). "Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis." *Toxicol Lett* **146**(3): 207-15.
- Winters, T. A., F. G. Febres, D. L. Fulgham, P. J. Bertics, T. M. Duello and J. Gorski (1993). "Ontogeny of the epidermal growth factor receptor during development of the fetal bovine mesonephros and associated organs of the urogenital tract." *Biol Reprod* **48**(6): 1395-403.
- Wolf, C. J., A. Hotchkiss, J. S. Ostby, G. A. LeBlanc and L. E. Gray, Jr. (2002). "Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study." *Toxicol Sci* **65**(1): 71-86.
- Wolf, C. J., G. A. LeBlanc and L. E. Gray, Jr. (2004). "Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat." *Toxicol Sci* **78**(1): 135-43.
- Wolf, C. J., G. A. LeBlanc, J. S. Ostby and L. E. Gray, Jr. (2000). "Characterization of the period of sensitivity of fetal male sexual development to vinclozolin." *Toxicol Sci* **55**(1): 152-61.
- Woodham, C., L. Birch and G. S. Prins (2003). "Neonatal estrogen down-regulates prostatic androgen receptor through a proteasome-mediated protein degradation pathway." *Endocrinology* **144**(11): 4841-50.
- Woodward, T. L., A. S. Mienaltowski, R. R. Modi, J. M. Bennett and S. Z. Haslam (2001). "Fibronectin and the alpha(5)beta(1) integrin are under developmental and ovarian steroid regulation in the normal mouse mammary gland." *Endocrinology* **142**(7): 3214-22.
- Wu, R. C., C. L. Smith and B. W. O'Malley (2005). "Transcriptional regulation by steroid receptor coactivator phosphorylation." *Endocr Rev* **26**(3): 393-9.
- Xavier, F. and S. Allard (2003). "Anti-Mullerian hormone, beta-catenin and Mullerian duct regression." *Mol Cell Endocrinol* **211**(1-2): 115-21.
- Xu, C. J. and D. Li (1998). "Pharmacokinetics of flutamide and its metabolite 2-hydroxyflutamide in normal and hepatic injury rats." *Zhongguo Yao Li Xue Bao* **19**(1): 39-43.
- Yamada, G., Satoh, Y., Baskin, L.S., Cunha, G.R. (2003). "Cellular and molecular mechanisms of development of the external genitalia." *Differentiation* **71**: 445-460.
- Yao, H. H., M. M. Matzuk, C. J. Jorgez, D. B. Menke, D. C. Page, A. Swain and B. Capel (2004). "Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis." *Dev Dyn* **230**(2): 210-5.
- Yao, H. H., W. Whoriskey and B. Capel (2002). "Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis." *Genes Dev* **16**(11): 1433-40.
- Yarbrough, W. G., V. E. Quarby, J. A. Simental, D. R. Joseph, M. Sar, D. B. Lubahn, K. L. Olsen, F. S. French and E. M. Wilson (1990). "A single base mutation in the androgen receptor gene causes androgen insensitivity in the testicular feminized rat." *J Biol Chem* **265**(15): 8893-900.
- Yeap, B. B., J. A. Wilce and P. J. Leedman (2004). "The androgen receptor mRNA." *Bioessays* **26**(6): 672-82.
- Yeh, S., M. Y. Tsai, Q. Xu, X. M. Mu, H. Lardy, K. E. Huang, H. Lin, S. D. Yeh, S. Altuwaijri, X. Zhou, L. Xing, B. F. Boyce, M. C. Hung, S. Zhang, L. Gan and C. Chang (2002). "Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues." *Proc Natl Acad Sci U S A* **99**(21): 13498-503.
- Yin, Y., C. Lin and L. Ma (2006). "MSX2 promotes vaginal epithelial differentiation and wolffian duct regression and dampens the vaginal response to diethylstilbestrol." *Mol Endocrinol* **20**(7): 1535-46.

- Yin, Y. and L. Ma (2005). "Development of the mammalian female reproductive tract." J Biochem (Tokyo) **137**(6): 677-83.
- Zhan, Y., A. Fujino, D. T. MacLaughlin, T. F. Manganaro, P. P. Szotek, N. A. Arango, J. Teixeira and P. K. Donahoe (2006). "Mullerian inhibiting substance regulates its receptor/SMAD signaling and causes mesenchymal transition of the coelomic epithelial cells early in Mullerian duct regression." Development **133**(12): 2359-69.
- Zheng, Z., C. Cai, J. Omwancha, S. Y. Chen, T. Baslan and L. Shemshedini (2006). "SUMO-3 enhances androgen receptor transcriptional activity through a sumoylation-independent mechanism in prostate cancer cells." J Biol Chem **281**(7): 4002-12.
- Zhu, Y. S., M. D. Katz and J. Imperato-McGinley (1998). "Natural potent androgens: lessons from human genetic models." Baillieres Clin Endocrinol Metab **12**(1): 83-113.
- Zimmerman, S. A., W. R. Clevenger, B. B. Brimhall and W. S. Bradshaw (1991). "Diethylstilbestrol-induced perinatal lethality in the rat. II. Perturbation of parturition." Biol Reprod **44**(4): 583-9.
- Zuo, Z., Y. K. Tam, J. Diakur and L. I. Wiebe (2002). "Hydroxypropyl-beta-cyclodextrin-flutamide inclusion complex. II. Oral and intravenous pharmacokinetics of flutamide in the rat." J Pharm Pharm Sci **5**(3): 292-8.

Androgen-Dependent Mechanisms of Wolffian Duct Development and Their Perturbation by Flutamide

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Androgens play a vital role in Wolffian duct (WD) development, but the mechanisms that underlie this are unknown. The present study used *in utero* exposure of pregnant rats to the androgen receptor antagonist flutamide (50 or 100 mg/kg) to explore possible mechanisms. Pregnant rats were treated from embryonic d 15.5 (E15.5), and WDs were isolated from fetuses from E17.5–E21.5 and from adults. WD morphology was evaluated, and total length of the duct lumen was determined in fetal samples. Fetal WDs were immunostained for androgen receptor and stromal (inner and outer) and/or epithelial-cell-specific markers and analyzed for cell proliferation and apoptosis. In adulthood, most flutamide-exposed males lacked proximal WD-derived tissues, whereas at E18.5–E19.5, a time when the WD has completely regressed in females, a complete normal WD was present in all flutamide-exposed animals. This suggests that flutamide, at doses of 50 or 100 mg/kg, interferes

with WD differentiation, not stabilization. Consistent with this, WD elongation/coiling increased in controls by 204% between E19.5 and E21.5 but increased less significantly (103%) in flutamide-exposed animals. This was associated with reduced cell proliferation, but there was no increase in apoptosis or change in expression of androgen receptor mRNA or protein. Flutamide treatment impaired differentiation of inner stromal cells, shown by decreased expression of smooth muscle actin, before effects were noted in the epithelium, consistent with androgens driving WD development via stromal-epithelial interactions. In conclusion, WD differentiation is far more susceptible to blockade of androgen action than is its initial stabilization, and these effects may be mediated by disruption of stromal-epithelial interactions. (*Endocrinology* 147: 4820–4830, 2006)

DURING MAMMALIAN DEVELOPMENT, the urogenital tract is identical in both sexes and is made up of two duct systems, the Müllerian duct and the Wolffian duct (WD) (1, 2). In males, Sertoli cells secrete anti-Müllerian hormone, which causes the Müllerian duct to degenerate, whereas Leydig cells secrete testosterone, which stabilizes and rescues the WD (2, 3). In females, the lack of androgens prevents stabilization of the WD, resulting in its degeneration (3). Once stabilized, the male WD begins to differentiate to form its adult derivatives; the cranial portion of the WD convolutes to form the adult epididymis, the central portion remains a relatively simple straight duct and forms the vas deferens, whereas the seminal vesicles bud off the distal segment (4, 5).

It is widely accepted that WD development is under the control of testosterone (2, 6), which is synthesized by the Leydig cells of the testis (7) and is delivered directly from the testis down the lumen of the WD (8). Testosterone secretion begins at around E15.5 in the fetal rat with a peak at E19.5 (9). In some cells, testosterone can be metabolized by 5 α -reductase to form the more potent androgen dihydrotestosterone (DHT) or aromatized to form estrogens (2). However, it is unlikely that either of these hormones are involved in

WD development because aromatase could not be detected in the fetal WD (unpublished findings) and estrogen receptor knockout mice and aromatase knockout mice have epididymides present in adulthood (10, 11). Furthermore, DHT has not been detected in the WD until after epididymal differentiation is complete (12, 13), and 5 α -reductase-deficient patients show normal WD differentiation (14), whereas rats exposed to finasteride, a 5 α -reductase inhibitor, show normal WD development (15).

Androgen action is mediated via the androgen receptor (AR), which is a member of the superfamily of ligand-activated steroid hormone receptors (16). The AR binds both testosterone and its metabolite DHT with high specificity and affinity; however, DHT dissociates less easily from the AR and is thus more effective at stabilizing the receptor in its active conformation (2, 17). Patients with mutations in the AR affecting its activity and/or expression exhibit a range of phenotypic abnormalities (18). In the case of complete androgen insensitivity, genetic XY males are born with a female phenotype, intraabdominal testes, no prostate, and a lack of WD-derived tissues (19–21).

Simple columnar epithelial cells line the lumen of the WD with stromal cells surrounding this epithelium. The AR is first expressed in the rat WD stroma at E16.5 and at low levels in the epithelial cells by E17.5 (22, 23). Studies in both male and female reproductive tracts show that mesenchymal cells can determine the morphological fate of the overlying epithelium, possibly via local production of growth factors (24–27). It has been proposed that stromal cells are the primary target for androgen action and that testosterone may induce proliferation and differentiation of epithelial cells through stromal-epithelial interactions (24, 28).

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Abbreviations: AR, Androgen receptor; DHT, dihydrotestosterone; E0.5, embryonic d 0.5; NGS, normal goat serum; SMA, smooth muscle actin; TBS, Tris-buffered saline; TBST, TBS containing 0.1% Tween 20; WD, Wolffian duct.

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Administration of the AR antagonist flutamide during pregnancy has been shown to dose-dependently impair masculinization. For example, treatment with 18 mg/kg flutamide resulted in complete feminization of external genitalia but normal WD differentiation (15). However, at doses above 100 mg/kg flutamide, the vas deferens was absent unilaterally or bilaterally, and only small remnants of the epididymis were present in adults (15). It is generally presumed that the latter effect results from interference with WD stabilization but this has not been shown directly during fetal life.

Although it is obvious that androgens play a role in WD development, the molecular and cellular events that underlie its stabilization and subsequent differentiation have received little attention to date. To address this, we have used a model system in which WD development can be altered, thus enabling the investigation of WD stabilization and differentiation with the aim of elucidating the cellular mechanisms responsible for androgen-dependent WD development. This is the first report to have taken this approach, and it was used to address the following specific questions. 1) Does flutamide-induced blockade of androgen action affect stabilization of the male WD, as occurs naturally in females, and/or its differentiation? 2) Do androgens control WD development by altering cell proliferation and/or apoptosis, and does this preferentially target one cell compartment? 3) Does altered expression of AR play a role in flutamide-induced inhibition of WD development? 4) Are the effects of flutamide treatment first detectable in the stromal compartment, consistent with the view that epithelial development is mediated by androgen effects on the stroma?

Materials and Methods

Animals

Wistar rats were bred and maintained in our own animal house under standard conditions according to United Kingdom Home Office guidelines. Animals had access *ad libitum* to water and a soy-free breeding diet (SDS, Dundee, UK). Time matings were set up, and the presence of a vaginal plug was taken as evidence of mating; this was defined as embryonic d 0.5 (E0.5).

In vivo treatment

Pregnant dams ($n = 70$) were randomly allocated to treatment groups and dosed daily by oral gavage between 0830 and 1000 h with flutamide (Sigma, Poole, UK) at 50 mg/kg ($n = 30$ dams) and 100 mg/kg ($n = 18$) in 1 ml/kg corn oil/2.5% dimethylsulfoxide (Sigma) or vehicle alone ($n = 22$). Dosing was undertaken from E15.5, the time at which the fetal testis begins androgen synthesis, until the day before cull. Flutamide doses were selected based on results previously reported (15). Dams were weighed daily and checked for signs of toxicity.

Dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Fetuses were recovered at E17.5–E21.5, decapitated, and placed in ice-cold PBS (Sigma). WDs were collected from male fetuses by microdissection, examined with a Leica MZ6 dissecting microscope (Leica Microsystems UK Ltd., Milton Keynes, UK) and photographed using a Leica ICA camera. For each fetus, one WD was snap frozen in liquid nitrogen, whereas the other was fixed in Bouin's for 1 h, transferred into 70% ethanol, and processed for 17.5 h in an automated Leica TP1050 processor. Fixed WDs were embedded horizontally in paraffin wax and processed and sectioned onto coated slides using standard procedures. Representative WDs from the aforementioned litters were subsequently used for the studies detailed below; at least three animals from at least three litters were studied per age/treatment group. Only samples that were analyzable were studied; for example,

WDs from flutamide-exposed animals with incomplete lumens (7% of animals at E21.5) were not included in the analysis of luminal length. Thus, results presented for luminal length are likely to underestimate the overall effect of flutamide exposure.

Gross morphology and histological analysis

Reproductive tracts from control and flutamide-exposed males and control females were analyzed microscopically, at the time of dissection, for any gross morphological abnormalities. Considerable variation was seen in the severity of malformation of the WDs after flutamide exposure; therefore, careful note was taken of any macroscopic abnormalities. Reproductive tracts from control female fetuses were also recovered, and the degree of regression of the WD was noted. Gross histological analysis was performed on WD sections stained with hematoxylin and eosin, using standard protocols.

WD luminal length measurement

Differentiation of WDs was quantified by measuring the luminal length of the epididymal segment of WDs from control and treated animals; a line was digitally drawn through the lumen of the WD image taken at the time of dissection using the Image J program (National Institutes of Health, Bethesda, MD). This line was drawn from the head of the epididymis, where the efferent ducts end, to the tail of the epididymal section just before the start of the vas deferens. To ensure reproducibility and to correct for individual variation, luminal length was measured for WDs from 15–37 animals from at least three different litters per treatment group.

Immunohistochemistry

Immunohistochemistry was performed on WDs recovered from control and treated fetuses at E17.5–E21.5 using standard avidin peroxidase protocols (see Ref. (29)). For AR, pan-cytokeratin, and cleaved caspase 3, antigen retrieval was performed using 0.01 M citrate buffer (pH 6.0). Sections were pressure cooked for 5 min at full pressure, left to stand for 20 min, and then cooled under running water. Endogenous peroxidase activity was blocked by washing sections in 3% H₂O₂ in methanol for 30 min at room temperature. All washes comprised two 5-min washes at room temperature in Tris-buffered saline (TBS) (0.05 M Tris-HCl, pH 7.4, and 0.85% NaCl). Nonspecific binding sites were blocked by incubating sections in normal goat serum (NGS) (Autogen Bioclear UK Ltd., Wiltshire, UK) diluted 1:4 in TBS containing 5% BSA (Sigma). Sections were incubated overnight at 4 °C with primary antibodies diluted in NGS/TBS/BSA: AR at 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA), smooth muscle actin (SMA, Sigma) at 1:10,000, pan-cytokeratin (Sigma) at 1:200, and cleaved caspase 3 (Cell Signaling Technology, Beverly, MA) at 1:200. Control sections were incubated with blocking peptide when available or blocking serum alone to confirm antibody specificity. Sections were incubated with the appropriate secondary antibody, either biotinylated goat antirabbit or biotinylated goat antimouse, diluted 1:500 in NGS/TBS/BSA for 30 min at room temperature before incubation for 30 min with avidin-biotin conjugated with peroxidase diluted in 0.05 M Tris-HCl (pH 7.4) according to the manufacturer's instructions (ABC-HRP; Dako, Ely, UK). Antibody localization was determined using 3,3'-diaminobenzidine (liquid DAB⁺; Dako) until staining was optimally detected in control sections; the reaction was stopped by immersing the sections in distilled water. Sections were counterstained in Harris's hematoxylin and mounted using Pertex (Cell Path, Hemel Hempstead, UK). Cellular sites of expression of AR, SMA, pan-cytokeratin, and cleaved caspase 3 were determined and slides photographed using a Provis AX70 (Olympus Optical, London, UK) microscope fitted with a Canon DS6031 camera (Canon Europe, Amsterdam, The Netherlands).

Fluorescence immunohistochemistry

To delineate stromal and epithelial compartments, fluorescence immunohistochemistry was used to colocalize three proteins in WDs recovered from control and treated fetuses at E17.5–E21.5. Antigen retrieval was performed as detailed above. All washes were two 5-min washes at room temperature in PBS (Sigma). At each stage, control sections were incubated with blocking serum without antibody to con-

firm antibody specificity. Nonspecific binding sites were blocked by incubating sections in NGS diluted 1:4 in PBS containing 5% BSA (Sigma). Sections were incubated overnight at 4°C with anti-pan-cytokeratin antibody (Sigma) diluted 1:200 in NGS/PBS/BSA and was detected by incubating sections for 1 h with goat antimouse secondary antibody directly conjugated with Alexa Fluor 488 (Molecular Probes, Poort Gebouw, The Netherlands) diluted 1:200 in PBS to produce green fluorescence. Sections were incubated for 30 min with mouse IgG (Sigma) diluted 1:2000 in NGS/PBS/BSA to block any remaining mouse IgG sites and prevent subsequent nonspecific binding. Nonspecific binding sites were blocked again by incubating sections with NGS/PBS/BSA for 30 min before incubating overnight at 4°C with anti-AR (Santa Cruz Biotechnology) diluted 1:50 in NGS/PBS/BSA. AR immunostaining was detected using biotinylated goat antirabbit IgG secondary antibody (Dako) diluted 1:500 in NGS/PBS/BSA for 30 min followed by incubation for 1 h with streptavidin-conjugated Alexa 546 (Molecular Probes), producing red fluorescence. Nonspecific binding sites were blocked again with NGS/PBS/BSA for 30 min before incubating overnight at 4°C with anti-SMA antibody (Sigma) diluted 1:500 in NGS/PBS/BSA. Sections were incubated for 1 h with goat antimouse labeled with Cy5 (Amersham Biosciences, Little Chalfont, UK) diluted 1:60 in PBS, producing blue fluorescence. Sections were counterstained for 10 min with a nuclear-specific blue fluorescent label, 4',6-diamidino-2-phenylindole (Sigma), diluted 1:1000 in PBS and then mounted in Mowiol mounting medium (Calbiochem, Lutterworth, UK). Fluorescent images were captured using a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

To ensure reproducibility of results and allow accurate comparison of immunostaining between treatment groups, sections of WDs from control and treated animals were processed in parallel on at least three occasions; sections of WDs from at least three animals in each treatment group were run on each occasion. Appropriate negative controls were included, whereby the primary antibody was replaced by blocking peptide or NGS alone, to ensure staining observed was specific; none of the antibodies used showed other than minor nonspecific staining.

Apoptosis analysis

Cleaved caspase 3 immunostaining was performed on WDs from control and flutamide-exposed fetuses using standard methods, as detailed above. Very few cleaved caspase-3-positive cells were detected; therefore, a detailed stereological analysis was not appropriate and all positive cells were manually counted in each WD using an Axiolab microscope (Carl Zeiss Ltd.).

Frequency of cell mitoses in WDs

To determine whether cell proliferation in WD compartments was affected by flutamide treatment, various cell cycle markers and analytical methods were investigated. For technical reasons, it was considered that determination of the proliferation index was impractical for the densely packed stromal cell compartment. Instead, a method was devised to allow enumeration of the total number of mitotic cells in each compartment, as outlined below.

WD sections from control and flutamide-exposed fetuses at E19.5–E21.5 were immunostained for phospho-histone H3 (Upstate Biotechnology, Dundee, UK) using a Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK) and a polymer high-contrast program. Briefly, after high-pressure antigen retrieval, slides were peroxidase blocked for 5 min and incubated for 2 h with the primary antibody diluted 1:1000 in the diluent supplied and then with the post-primary reagent for 15 min. Control sections were incubated with diluent alone to confirm antibody specificity. Sections were then incubated with the polymer reagent for 15 min to increase sensitivity of detection before DAB detection for 10 min, counterstained in hematoxylin for 5 min, dehydrated, and mounted as detailed above.

Phospho-histone H3-positive cells were counted in the epithelial compartment and the inner and outer stromal layers of the WD using the $\times 20$ objective on an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK). Image-Pro Plus version 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, UK) was used for analysis. Positive cells were counted only in the future epididymal portion of the WD, not

in the efferent ducts or vas deferens. Because differences were noted in the degree of coiling along the length of the future epididymal portion of the WD, phospho-histone H3-positive cells were initially counted in each region of the future epididymis individually, defined as in the adult epididymis as caput, corpus, and cauda (5) (Fig. 1D). However, no consistent difference was seen in phospho-histone H3 staining between the different regions of the epididymal portion of the WD; therefore, proliferation was subsequently analyzed in the epididymal portion of the WD as a whole.

Variation was noted in the length of the WD visible in each section as well as the amount of epithelium visible per section; this was a result of both treatment effects and the plane of sectioning. To correct for this variation, the overall length of the WD and the length of epithelium visible in each section were measured. The number of phospho-histone H3-positive cells in the epithelial compartment was then divided by the total length of epithelium visible in the section to calculate proliferation per micron of epithelium. Because flutamide exposure reduces coiling and WD luminal length, it was important to take this into account when analyzing cell proliferation. The number of positive epithelial cells per micron of epithelium was therefore multiplied by the luminal length for that specific WD (calculated at the time of dissection, method detailed above) to calculate total epithelial proliferation in the whole epididymal portion of the WD, not just cell proliferation in the section being analyzed. Similarly, the number of phospho-histone H3-positive cells in the stromal compartment was divided by the length of WD epithelium visible in the section to calculate stromal proliferation per micron of stroma and hence correct for variation in the length of the WD. This was then multiplied by the luminal length of the WD to calculate total stromal proliferation in the whole epididymal portion of the WD. Sections from eight to 14 fetuses from three to five separate litters were analyzed from both control and flutamide-exposed animals at each age. To confirm that this analysis was likely to reflect the cell proliferation/mitotic index, the total number of phospho-histone H3-positive and negative epithelial cells were counted (500–700 cells) in three control and three F100 E21.5 WDs, and an epithelial mitotic index was derived (percent phospho-histone H3-positive cells/total number of cells \times 100%).

Epithelial cell height measurement

WD sections from six control fetuses and six fetuses from each of the flutamide treatment groups at E21.5 were randomly selected and immunostained for pan-cytokeratin as detailed above to clearly label all epithelial cells. Using a $\times 63$ objective, epithelial cell height was measured in every fifth epithelial cell per section, using the software and stereological equipment noted above. This was performed separately for the caput, corpus, and cauda regions of the future epididymal portion of the WD. Only epithelial cells in which the nucleus could be clearly identified were measured, thus excluding from analysis any epithelial cells from the flutamide treatment groups that were severely flattened or disintegrating. In a subset of these animals, epithelial cell width was also measured using the same method.

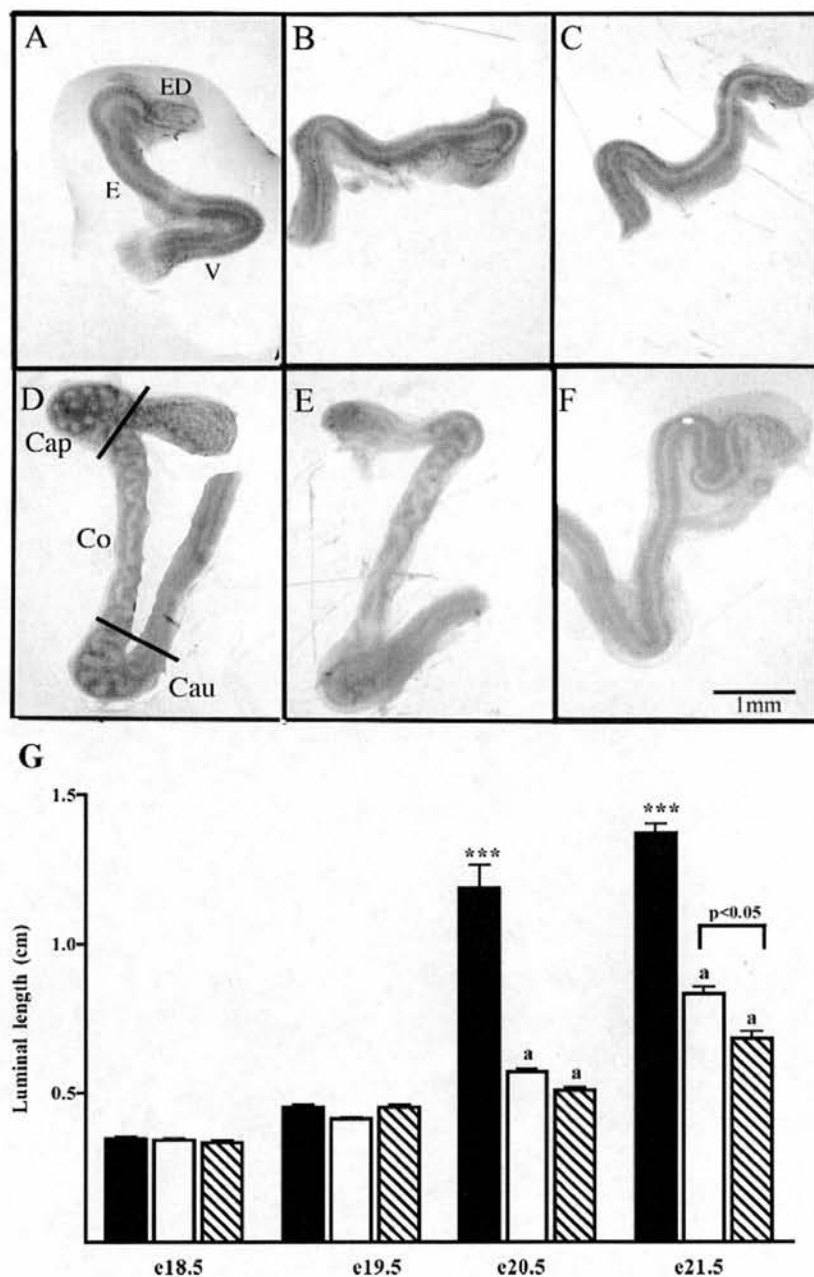
Quantitative RT-PCR

RNA was isolated from frozen WDs using the RNeasy Mini extraction kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. RNA was DNase treated during extraction using RNase-free DNase on the column digestion kit (QIAGEN), and random hexamer primed cDNA was prepared using the Applied Biosystems TaqMan RT kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed with the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Expression of AR mRNA was determined using the Assay-On-Demand Gene Expression system for rat AR (Rn00560747_m1; Applied Biosystems). The expression level of AR mRNA was corrected using an internal control, 18S rRNA, and related to rat uterus expression levels. Results shown are the mean of a minimum of three WDs per treatment group performed in triplicate on at least two occasions.

Western blot analysis

Protein was harvested from frozen WDs using RIPA [1% Triton X-100, 15 mM HEPES-NaOH (pH 7.5), 0.15 mM NaCl, 1% sodium deoxycholate,

FIG. 1. Age-dependent increase in coiling/elongation of the WD between E18.5 and E21.5. Representative WDs recovered from control fetuses (A and D) and fetuses exposed to 50 mg/kg flutamide (B and E) or 100 mg/kg flutamide (C and F) at E18.5 (top) and E21.5 (bottom) are illustrated. Note the reduced coiling in WDs from flutamide-exposed animals at E21.5 (E and F) when compared with the age-matched control (D). Note the demarcation of the epididymal regions of a control WD at E21.5 (D) into the caput (Cap), corpus (Cor), and cauda (Cau), as in the adult epididymis. Scale bar, 1 mm. E, Prospective epididymis; ED, efferent ducts; V, prospective vas deferens. The graph (G) illustrates quantification of coiling (luminal length) of the WDs. Note the progressive increase in luminal length of WDs from controls between E18.5 and E21.5 (black bars). In WDs from animals exposed *in utero* to either 50 mg/kg (white bars) or 100 mg/kg (striped bars) flutamide, there was a significant reduction in luminal length at E20.5 and E21.5 when compared with controls, whereas at E18.5 and E19.5, no difference was evident. Values are mean \pm SEM for 15–36 animals per group. ***, $P < 0.001$ in comparison with respective value for preceding day; a, $P < 0.001$ in comparison with respective control value.



0.1% SDS, 1 mM sodium orthovanadate, 10 mM EDTA, and 0.5% protease inhibitor cocktail (Sigma)] lysis buffer. WDs were homogenized in 75 μ l RIPA buffer and then incubated on ice for 1 h. Samples were centrifuged at 2500 rpm for 10 min and the supernatant collected. The protein concentration was determined using a Bio-Rad BCA protein assay kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hemel Hempstead, UK). Proteins were denatured by boiling with SDS loading buffer for 5 min before loading 20 μ g of each protein extract onto a 7.5% polyacrylamide gel. Gels were subjected to electrophoresis at 100 V under reducing conditions for 2 h. The proteins were electrophoretically transferred overnight at 20 V onto nitrocellulose membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were washed in TBS containing 0.1% Tween 20 (TBST) (Sigma) before incubating in TBST with 5% milk for 1 h at room temperature to block nonspecific binding sites. Membranes were washed twice in TBST for 5 min and then incubated overnight at 4 $^{\circ}$ C with anti-AR antibody (Santa Cruz Biotechnology) diluted 1:200 in TBST, anti-SMA antibody (Sigma) diluted 1:10,000, or an anti-

β -tubulin antibody (Sigma) as a standardization control at 1:300 in TBST. After washing with TBST, membranes were incubated for 1 h at room temperature with antirabbit horseradish peroxidase or antimouse horseradish peroxidase (SAPU, Carlisle, UK), respectively, at 1:5000 in TBST/5% milk. The signal was detected using ECL plus Western blot detection reagents according to the manufacturer's instructions (Amersham Biosciences). Signals were visualized using high-performance chemiluminescence imaging film (Amersham Biosciences) and developed using a Xograph compact $\times 4$ imaging system (Xograph, Tetbury, UK). Antibody specificity was confirmed by the detection of only one band at the expected size when visualizing each antibody. The intensity of the bands was quantified using the Typhoon 9400 variable mode imager (Amersham Biosciences). The expression level of protein was corrected for loading using β -tubulin and related to E19.5 control protein levels. To ensure reproducibility of results, the Western blot was performed three to six times, and protein was isolated from at least three WDs from three different litters each time.

Statistical analysis

Values have been expressed as means ± SEM. Data were analyzed using one-way ANOVA followed by the Bonferroni *post hoc* test, using GraphPad Prism version 4 (Graph Pad Software Inc., San Diego, CA).

Results

Normal development of the WD

Figure 1 shows images of WDs isolated at E18.5 and E21.5. At E18.5 (Fig. 1A), the epididymal portion of the WD is a simple, straight uncoiled duct. Coiling first becomes evident at the cranial and caudal ends of the future epididymal portion of the WD at E20.5, whereas the corpus remains relatively uncoiled (not shown). By E21.5, the full length of the epididymal portion of the WD appears highly convoluted, including the corpus (Fig. 1D). This age-dependent increase in coiling and development was confirmed quantitatively by the demonstration of an increase in luminal length (Fig. 1G). No significant increase ($P > 0.05$) in WD luminal length was seen between E18.5 and E19.5, but a highly significant increase was noted on consecutive days thereafter.

Impact of flutamide treatment on gross WD morphology

Consistent with previous studies (30) that have blocked androgen action *in utero*, considerable variation between animals was noted in the degree of WD coiling after exposure to flutamide; this was more apparent at later fetal ages (E20.5–E21.5) when WDs are more differentiated in fetuses from control mothers. To take account of this variability, WDs were analyzed from 19–37 animals from at least three litters per age/treatment group.

As in control WDs, an age-dependent increase was noted in the degree of coiling and hence differentiation of WDs from flutamide-exposed fetuses, but the degree of coiling was reduced compared with age-matched controls (Fig. 1). As in controls, no coiling was seen in WDs from flutamide-exposed animals at E18.5 (Fig. 1, B and C). At E20.5 and E21.5, but not at E18.5 or E19.5, flutamide-exposed animals showed a highly significant reduction in WD coiling and luminal length compared with controls (Fig. 1G). At E21.5, coiling was more dramatically reduced in fetuses exposed to 100 mg/kg flutamide than those exposed to 50 mg/kg (Fig. 1, F compared with E, respectively). As well as this reduction in coiling, some WDs from flutamide-exposed fetuses appeared incomplete at E21.5 with thinning of the epithelium and missing corpus segments; this was not seen in control WDs. Missing corpus segments were first noted at E21.5 whereby 5 and 11% of WDs recovered from animals exposed to low- and high-dose flutamide, respectively, had incomplete lumens (Table 1). These fetuses were from the same litter within each treatment group. This was not seen in WDs at E18.5–E20.5 from flutamide-exposed animals. This is in contrast to the natural regression of WDs in females, in which by E18.5 the WD has fully regressed and is no longer visible (Fig. 2B).

Impact of fetal exposure to flutamide on male reproductive tract morphology

Exposure to either dose of flutamide prevented normal masculinization of the fetal external genitalia, with anogeni-

TABLE 1. Number of incomplete WD in control and flutamide-exposed animals

Age	Treatment	Number of incomplete WDs (%)	Number of complete WDs
E18.5	Control	0	10
	Flutamide 50 mg/kg	0	21
	Flutamide 100 mg/kg	0	21
E19.5 ^a	Control	0	37
	Flutamide 50 mg/kg	0	47
	Flutamide 100 mg/kg	0	35
E20.5	Control	0	25
	Flutamide 50 mg/kg	0	68
	Flutamide 100 mg/kg	2 (7) ^b	29
E21.5	Control	0	24
	Flutamide 50 mg/kg	2 (5) ^b	39
	Flutamide 100 mg/kg	4 (11) ^b	34
Adults	Control	0	18
	Flutamide 100 mg/kg	10 (83) ^c	2

^a At E19.5, the WD in control female rats has completely regressed.
^b Note that these WDs were all from the same litter (E20.5 F100, one of three; E21.5 F50, one of six; and E21.5 F100, one of three).
^c Note that these reproductive tracts were found in males from three of three litters.

tal distance reduced in E21.5 flutamide-exposed males to a length similar to that of control females (data not shown). Flutamide-exposed E21.5 males had no prostate and a vaginal pouch was present, but testis descent was largely unaffected (data not shown). Consistent with previous reports (31–33), these abnormalities were still evident postnatally. Considerable variation was noted in the degree of abnormality of WD-derived adult tissues, including underdeveloped or incomplete epididymides, lack of vas deferens, and underdeveloped or vestigial seminal vesicles (data not shown). As in the fetus, the corpus segment of the epididymis was often absent with 83% of epididymides studied being incomplete (Table 1). In five animals, there was near-complete absence of all epididymal segments and the vas deferens.

Histology

The WD is composed of two cellular compartments, simple columnar epithelial cells lining the lumen of the duct and stromal cells surrounding this epithelium, and this did not

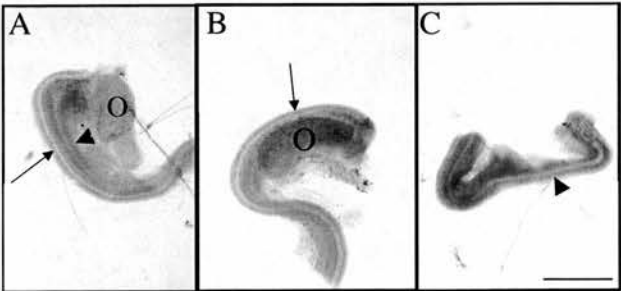


FIG. 2. Natural regression of the WD in female rats between E17.5 (A) and E18.5 (B) in comparison with the effect of flutamide (100 mg/kg) treatment on WDs in the male (C). Note the presence of both the Müllerian duct (arrow) and WD (arrowhead) in the female at E17.5 (A), whereas the WD has regressed by E18.5 (B). In comparison, in the flutamide-exposed male at E18.5 (C), the WD is still present and is comparable to its age-matched control (see Fig. 1A). Scale bar, 1 mm.

vary along the length of the WD or with age. The mesenchymal cells can be easily separated into two distinct populations, those directly surrounding the epithelial cells, which are more densely packed and termed here as inner stroma, and those that make up the rest of the body of the WD, referred to here as outer stroma. This can easily be seen by hematoxylin and eosin staining (Fig. 3A), but this distinction was confirmed by staining for SMA, which is expressed only in the inner and not the outer stromal cells of the WD (Fig. 3B). SMA protein expression switched on in these cells between E17.5 and E18.5 and increased dramatically between E18.5 and E19.5 (data not shown).

Examination of WDs revealed no obvious histological differences between WDs from control and flutamide-exposed fetuses at E18.5 or E19.5 (data not shown). However, at E21.5 after flutamide exposure (high and low dose), the WD epithelium often looked abnormal compared with that of controls, appearing more flattened and sometimes fibroblast-like. The extent of this flattening varied between WDs but was not seen in controls or flutamide-exposed WDs at any earlier age.

AR expression

AR was immunolocalized to cell nuclei throughout the WD at all ages examined (E17.5–E21.5). Stromal cells directly surrounding the epithelium showed the most intense immunostaining, whereas the epithelial cells showed weaker staining (Fig. 4). WDs from flutamide-exposed animals displayed a similar pattern of AR expression to controls at all ages studied with AR expressed in the stroma by E16.5 and epithelial expression switching on in some cells by E17.5 (data not shown). Additional analysis using Western blotting and TaqMan quantitative real-time PCR confirmed that there was no significant difference in either AR protein or mRNA expression in control compared with flutamide-exposed WDs at E17.5–E21.5 (Fig. 4, F and G). Variation was seen in

the levels of AR mRNA and protein within each age/treatment; however, this was not significant and presumably represents variation between individual WDs.

Role for apoptosis in flutamide-induced impairment of WD development

Apoptotic cells were rarely detected (typically one cell per section) in WDs from either control or flutamide-exposed fetuses at any age studied (data not shown).

Frequency of cell mitoses in WDs

Cell mitosis was evident in all compartments of WDs from control and flutamide-exposed animals at all ages (Fig. 5, A and B). Immunostaining for phospho-histone H3 suggested fewer mitotic cells were present in WDs from flutamide-exposed fetuses compared with controls, and this was confirmed by quantitation that showed a significant reduction in numbers of mitotic epithelial and stromal cells per WD compared with controls (Fig. 5). This reduction was seen at all ages studied; however, results are presented here only for E21.5. Cell proliferation data are expressed as the overall number of mitotic cells in the complete epididymal portion of the WD rather than per 100 μ m epithelium so as to take into account the treatment-induced reduction in WD length. If this correction was not applied, a similar treatment-induced reduction in cell mitosis was evident but was of smaller magnitude.

Analysis of the epithelial mitotic index yielded comparable results, with epithelial mitosis reduced ($P < 0.05$) in flutamide-exposed WDs ($0.2 \pm 0.03\%$, mean \pm SEM; $n = 3$), compared with control WDs ($0.3 \pm 0.02\%$, mean \pm SEM; $n = 3$), indicating that the analytical method used reflects the mitotic index.

Reduction in epithelial cell height after flutamide exposure

Upon visual inspection, areas of the epithelial compartment in some animals at E21.5 appeared abnormal after flutamide exposure, with shorter epithelium, missing cells, and widened lumens (Fig. 6B); this was not seen in controls (Fig. 6A). This difference was confirmed quantitatively at E21.5 as flutamide exposure reduced WD epithelial cell height (Fig. 6C). This reduction was more dramatic in the corpus and caudal portions of the WD and was most pronounced in animals exposed to 100 mg/kg flutamide. No significant difference was noted in the width of WD epithelial cells in flutamide-exposed animals compared with controls (data not shown).

Evidence for earliest effects of flutamide treatment on inner stromal cells

Because the data presented above indicated an impact of flutamide treatment on both stromal and epithelial tissues of the WD, we sought evidence of stromal-specific effects using SMA, a stromal cell marker. Immunostaining suggested a reduction in this SMA-positive layer in flutamide-exposed WDs compared with age-matched controls, although this varied considerably between WDs. This decrease was confirmed quantitatively by Western blot analysis, which re-

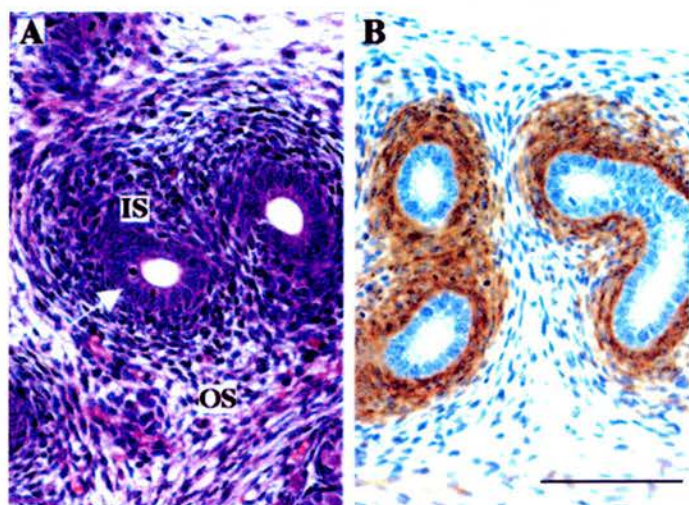


FIG. 3. Demarcation of the E21.5 WD stroma into the inner and outer stromal compartments (IS and OS, respectively). Note that the IS can be identified as the more dense compartment immediately proximal to the epithelium (arrow; A). This regionalization was confirmed by immunostaining for SMA, which is expressed only in the inner stroma (brown; B). Scale bar, 100 μ m.

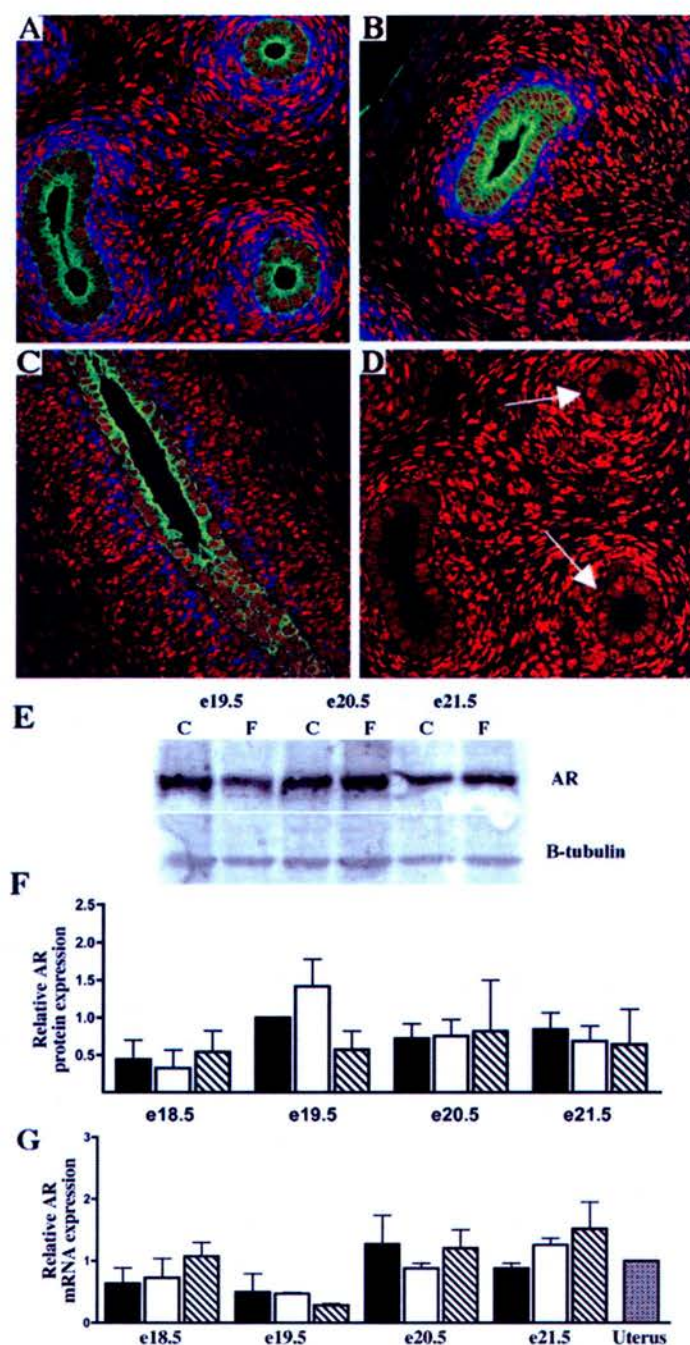


FIG. 4. AR expression in the different compartments of the WD caput at E21.5. Note that the inner stroma is identified by SMA immunostaining (blue) and the epithelium by cytokeratin staining (green). No obvious difference was seen in AR expression between WDs from control animals (A) and animals exposed to 50 mg/kg (B) or 100 mg/kg (C) flutamide. Image D shows AR only staining from Fig. 5A, highlighting that AR protein was expressed (red) more intensely in the stroma than in the epithelium (arrows) of the control WD at E21.5 (D), and flutamide treatment had no obvious effect on this pattern (B and C). Scale bar, 50 μ m. E, Representative Western blot for AR protein expression in WDs at E17.5–E21.5 ($n = 3$ WDs per age/treatment) with its loading control below. F, Quantitative analysis (mean \pm SEM) of AR protein from Western blots confirming no significant change in AR expression in WDs at E17.5–E21.5 from control (black bars) and flutamide-exposed (white bars, 50 mg/kg; striped bars, 100 mg/kg) rats ($n = 6$ Western blots, each using a pool of three WDs per age/treatment from at least three litters). Protein expression was

vealed that the biggest reduction was at E19.5 (Fig. 7), a time point at which no epithelial damage or change in luminal length was detectable in flutamide-exposed animals (Fig. 1).

Discussion

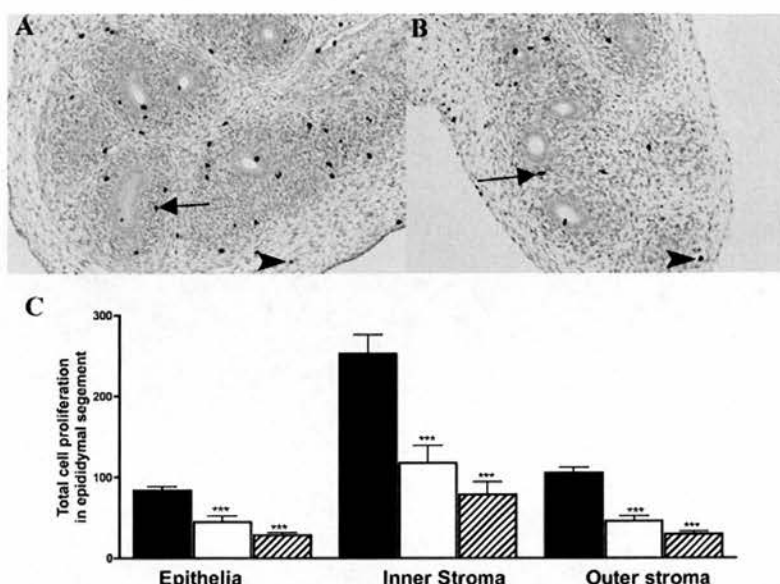
It is widely accepted that androgens play a vital role in WD development, but little is known about the molecular and cellular events that underlie this (2, 34, 35). The present study set out to use an *in utero* model system in which epididymal development can be altered to investigate the cellular mechanisms responsible for androgen-dependent WD development and allow better understanding of the mechanisms behind epididymal abnormalities in adults after reduced fetal androgen action. The main findings of these studies are, first, that *in utero* exposure to 50 or 100 mg/kg flutamide inhibits development, elongation, and differentiation of the WD but not its stabilization, contrary to previous interpretations. Second, these flutamide effects are mediated by effects on cell mitosis, but not apoptosis, and do not involve altered AR expression. Last, flutamide treatment appears to impair/delay differentiation of inner stromal cells at a stage in development before effects on the epithelium, consistent with the view that androgens drive WD development via stromal-epithelial interactions.

In males, the WD differentiates during neonatal life to form the epididymis, vas deferens, and seminal vesicles (4). In female rats, the WD regresses naturally between E16.5 and E18.5; this is believed to be because of a lack of androgens, whereas in males at this age, androgen action is believed to stabilize the WD, allowing it to subsequently differentiate into its adult derivatives (2, 3). In our rat colony, differentiation is first evident in the future epididymis by E20.5, suggesting that the developmental window for WD differentiation is between E19.5 and birth, coinciding with the peak in testosterone seen in male rats at E19.5 (9). This is in agreement with timings seen in previous studies (36).

It has been suggested that the pattern of AR expression reflects the androgen responsiveness of the tissue (23). We have mapped the AR to the epithelia from E17.5 and stroma of the WD from E16.5; however, epithelial AR expression was less intense than in stromal cells, particularly before E20.5. This is in agreement with other studies using antibodies demonstrating that ARs are expressed in the nuclei of both the stroma and epithelial cells of the rat WD by E17.5 (23). However, studies in mice using [3 H]dihydrotestosterone steroid autoradiography reported stromal expression from E13, but epithelial expression was not detected until E19, much later than in rats (37). This difference in timing of expression may be because of species differences or different methodologies. The more intense immunoexpression of AR in the stroma compared with the epithelium would be consistent with the view that androgen effects on early WD development occur mainly through stromal AR and that androgens stimulate the stroma to signal to the epithelium to indirectly

corrected for loading and expressed relative to levels in E19.5 control WD. G, No significant difference is shown in AR mRNA expression between control and flutamide-exposed WD at E18.5–E21.5, as determined by TaqMan quantitative real-time PCR (means \pm SEM; $n = 3$ –5 WDs per age/treatment, each from different litters).

FIG. 5. Frequency of mitotic cells (immunopositive for phospho-histone H3; black staining) in representative WDs from a control (A) and an animal exposed *in utero* to 50 mg/kg flutamide (B) at E21.5. Note the numerous mitotic cells in the inner stroma (arrows), outer stroma (arrow-head), and epithelium. Panel C shows the total number of mitotic epithelial and stromal cells in the entire epididymal segment of the WD at E21.5 in controls (black bars) and animals exposed to 50 mg/kg (white bars) or 100 mg/kg (striped bars) flutamide. ***, $P < 0.001$ in comparison with respective control value. Values are means \pm SEM for 12–16 animals.



control epithelial fate (24). Flutamide exposure did not alter AR expression; therefore, WDs from flutamide-exposed animals retained the potential for AR-mediated androgen ac-

tion. This conclusion is in contrast to findings by others who were unable to detect AR protein by immunohistochemistry in WDs from E21.5 fetuses exposed to 100 mg/kg flutamide (23). This difference is unexplained, although it could be because of rat strain difference or to the use of different anti-AR antibodies.

Previous studies have reported that flutamide exposure *in utero* results in abnormal epididymides in adults, but in these studies, it was not possible to identify whether the WD formed and stabilized but segments were later lost during differentiation or whether it failed to form and/or stabilize (31–33). In the present studies, WDs from flutamide-exposed males were present at E18.5 and E19.5 and appeared normal in all animals, with no obvious morphological differences or reduction in luminal length compared with their age-matched controls. This suggests that flutamide-exposed WDs initially form normally and are morphologically stabilized; however, it is unknown whether these WDs are functional at the biochemical level, and this needs additional investigation. In the regressing female WD, apoptosis is apparent, suggesting that the WD is dying rather than not differentiating (unpublished findings) (38, 39). In contrast, minimal cell apoptosis was detected in WDs from flutamide-exposed males at any age studied. Together these findings suggest that the doses of flutamide administered do not completely block the AR and so may not reduce androgen action sufficiently to impair WD stabilization and so cause it to regress, as occurs in the female (38). This difference could reflect the higher levels of testosterone reported to be available at E18.5 when the WD is undergoing stabilization than at E20.5 when the WD is differentiating (40), thus making it more difficult for flutamide to block androgen action. Alternatively, WD stabilization may require relatively low levels of androgens, less than that required for differentiation, or WD stabilization and differentiation may be regulated by different mechanisms. This is the first report that we are aware of highlighting WD development as a two-phase process, each of which may be differentially controlled. Insight from patients with complete androgen insensitivity syn-

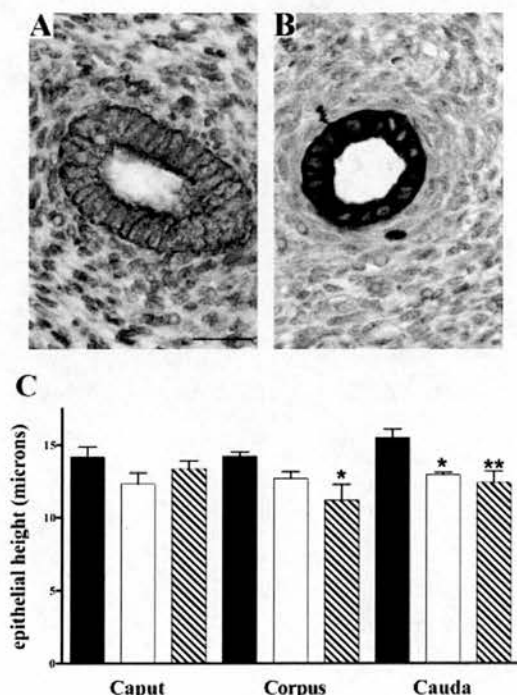
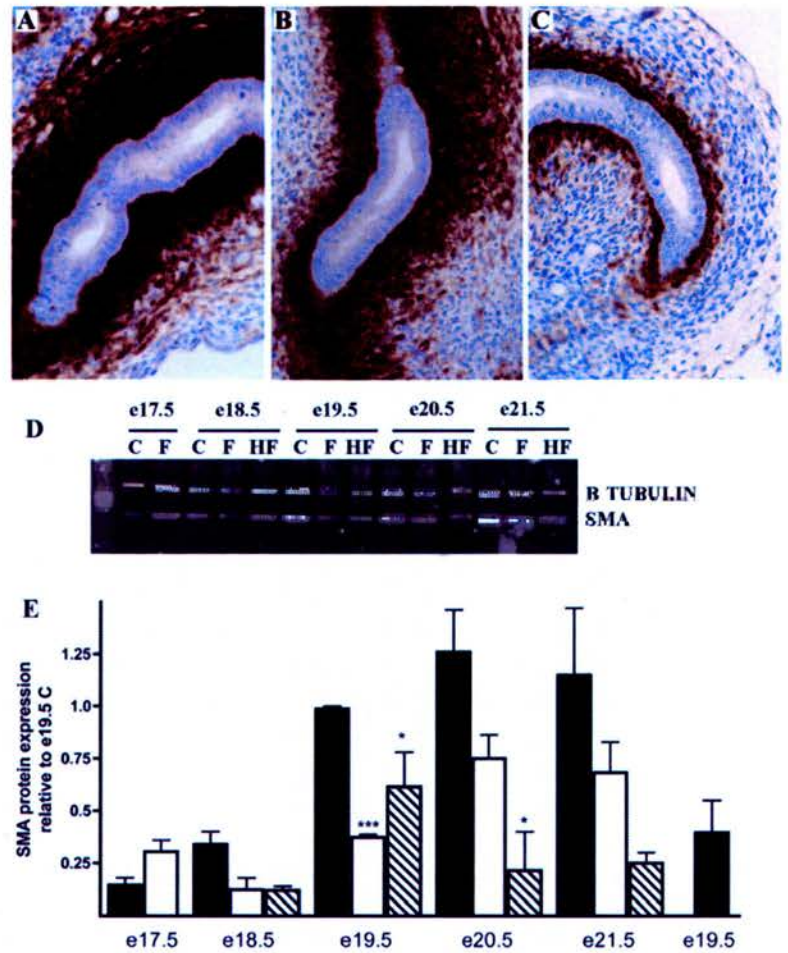


FIG. 6. Epithelial cell height at E21.5 in WDs from controls (A and black bars in C) and animals exposed to 50 mg/kg (B and white bars in C) or 100 mg/kg (striped bars) flutamide. Note that the epithelium (immunostained for cytokeratin) is flatter in WDs in the flutamide-exposed animal. Images in A and B are both from the caput region of a WD, the segment of the WD where epithelial height is least reduced by flutamide exposure. Scale bar, 25 μ m. Panel C shows quantification of epithelial cell height in WDs from controls (black bars) and from animals exposed to either 50 mg/kg (white bars) or 100 mg/kg (striped bars) flutamide. Note that epithelial cell height is reduced more in the corpus and caudal portions of the future epididymis after flutamide treatment. *, $P < 0.05$; **, $P < 0.01$ in comparison with respective control value. Values are means \pm SEM for five to seven animals per treatment from at least three different litters.

FIG. 7. Comparative expression of SMA at E19.5 in WDs from controls (A) and animals exposed *in utero* to 50 mg/kg (B) or 100 mg/kg (C) flutamide. Note the reduction in SMA expression in WDs after flutamide exposure. D, Representative Western blot for SMA protein in WDs from control (C) and flutamide-exposed animals (F, 50 mg/kg; HF, 100 mg/kg) at E17.5–E21.5 ($n = 3$ –5 WDs per age/treatment from at least three different litters). Testes (T; E21.5) were used as a positive control for SMA expression. E, Quantitative data expressed relative to SMA expression in control WDs at E19.5 (corrected for loading using β -tubulin). Note the increase in SMA protein expression between E18.5 and E19.5 in controls and the decrease in SMA expression after flutamide exposure (white bars, 50 mg/kg; striped bars, 100 mg/kg) compared with controls (black bars). *, $P < 0.05$; ***, $P < 0.001$ in comparison with respective control value. Values are means \pm SEM for two to three Western blots, each using a pool of three WDs per age/treatment from at least three litters.



drome and testicular feminization mice might further elucidate this as AR signaling is blocked in each case. Reports have shown that in both instances, adults lack any WD-derivatives (41, 42), however, no definitive evidence is published regarding the status of the fetal WD.

In controls, WDs showed a significant age-dependent increase in coiling and differentiation, as determined by quantification of luminal length; this is believed to be an androgen-dependent process. Unsurprisingly, flutamide interrupted this differentiation, thus confirming the vital role for androgens in differentiation of the stabilized WD. The most obvious explanation for this reduction in WD development could be an increase in cell apoptosis and/or a decrease in cell mitosis. Because apoptosis was minimal in WDs from flutamide-exposed animals, it was considered likely that altered cell proliferation was responsible. This is the first study to report the impact of androgen blockade on cell mitosis in the differentiating WD, showing androgen regulates both stromal and epithelial cell proliferation but that the consequences of reduced AR signaling can be noted earlier in the stromal compartment.

At E19.5, WDs from all flutamide-exposed animals were morphologically intact, suggesting that AR-mediated signaling was incompletely blocked fetally, even at 100 mg/kg flutamide, thus allowing WD rescue. Conversely, in adults that had been similarly exposed to flutamide *in utero*, the

majority of the WD-derived tissues were largely absent. This is in agreement with previous studies showing that exposure to antiandrogenic compounds *in utero* results in a high frequency of epididymal malformations in adult rats (15, 31–33, 43). The contrast between WD abnormalities at E19.5 and in adulthood in flutamide-exposed animals demonstrates that the major effect of flutamide is on epididymal differentiation rather than stabilization, contrary to what has been presumed to occur by earlier researchers (15, 33). The increased prevalence of epididymal abnormalities as development proceeds may be a result of failure to establish normal patterning of the WD fetally. Interfering with androgen action within a critical window of development can therefore impair WD patterning and hence differentiation into its adult derivatives, resulting in irrecoverable malformation of the reproductive tract and likely impairment of fertility.

By E21.5, WDs from flutamide-exposed animals show abnormalities in both gross morphology and histology, including shorter, flatter epithelia, occasional missing corpus segments of the future epididymis, reduced coiling, and incomplete lumens. This is not seen until E21.5; thus the epithelium initially forms normally in flutamide-exposed animals but degenerates during differentiation, possibly because of interrupted androgen-driven signaling between the stroma and epithelium. In multiple organ systems, it is believed that steroid hormones control the fate of epithelial

cells via interactions with the underlying stroma, and hormone withdrawal can result in epithelial-mesenchymal transformation (24, 26, 44–46). Because the epithelium in incomplete regions often appeared flattened with some cells even looking more fibroblast-like, it may be that a lack of androgen signaling resulted in dedifferentiation of some epithelial cells into mesenchymal-like cells, thus contributing to the impaired development of the WD. These missing segments were noted only in the corpus segment, the region of the WD that coils last and the segment of the adult epididymis that is least coiled. The androgen signal may be weaker in the corpus than in the caput or cauda, and thus flutamide treatment could have more impact on its development. The caput is closest to the testis and is therefore likely to be exposed to high levels of locally delivered androgens, but it is not obvious why the cauda should be less affected by flutamide treatment than the corpus. It is possible that the cauda may obtain testosterone from the blood as well as directly from the testis. However, there is unlikely to be a role for the more potent androgen DHT, because Wilson and Lasnitzki (12) and Siiteri and Wilson (13) both reported that DHT was not detectable in the WD until after epididymal differentiation was complete.

Blocking androgen action by flutamide exposure impaired stromal differentiation as indicated by reduced expression of SMA, an androgen-responsive stromal differentiation marker (47). This reduction in smooth muscle differentiation may explain the reduction in WD coiling seen after flutamide exposure. The flutamide-induced reduction in SMA expression was noted as early as E19.5, before any obvious signs of impaired epithelial development were apparent. This is consistent with previous studies in various organ systems that have shown that the stromal cells, via paracrine interactions, play a critical role in controlling epithelial proliferation, differentiation, and development (25, 26, 48–50). The mechanisms underlying these paracrine interactions are poorly understood, but growth factors and/or the extracellular matrix are likely to be involved.

There is substantial literature on the effects of reduced androgen action on the male reproductive tract using knockout mice and AR antagonists; however, most have studied the effects in adult males, and few have looked at how this impacts on development in fetal life. Because interfering with androgen action during fetal life, using 50 or 100 mg/kg flutamide, did not prevent stabilization of the WD but impaired its subsequent convolution and differentiation into its adult derivatives, the current study has highlighted WD development as a two-phase process with WD stabilization apparently requiring lower androgen action than WD differentiation. Why WD stabilization is not affected by administration of high doses of flutamide, approximately 5-fold higher than levels that induce complete agenesis of the prostate and feminization of the external genitalia, is something of a mystery and merits additional study. Future studies will also focus on using the present model system to investigate possible signaling pathways involved in WD patterning and differentiation. This should allow a clearer insight into the mechanisms behind interrupted epididymal development and function.

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References

1. Drews U 2000 Local mechanisms in sex specific morphogenesis. *Cytogenet Cell Genet* 91:72–80
2. George FW, Wilson J 1994 The physiology of reproduction. 2nd ed. New York: Raven
3. Huhtaniemi I 1994 Fetal testis: a very special endocrine organ. *Eur J Endocrinol* 130:25–31
4. Wilson JD, George FW, Griffin JE 1981 The hormonal control of sexual development. *Science* 211:1278–1284
5. Robaire BaH L 1988 Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: *Physiology of reproduction*. 2nd ed. New York: Raven Press
6. Veyssiere G, Berger M, Jean-Faucher C, de Turkheim M, Jean C 1982 Testosterone and dihydrotestosterone in sexual ducts and genital tubercle of rabbit fetuses during sexual organogenesis: effects of fetal decapitation. *J Steroid Biochem* 17:149–154
7. Nef S, Parada LF 2000 Hormones in male sexual development. *Genes Dev* 14:3075–3086
8. Tong SY, Hutson JM, Watts LM 1996 Does testosterone diffuse down the Wolffian duct during sexual differentiation? *J Urol* 155:2057–2059
9. Warren DW, Haltmeyer GC, Eik-Nes KB 1972 Synthesis and metabolism of testosterone in the fetal rat testis. *Biol Reprod* 7:94–99
10. Hess RA, Bunick D, Lubahn DB, Zhou Q, Bouma J 2000 Morphologic changes in efferent ductules and epididymis in estrogen receptor- α knockout mice. *J Androl* 21:107–121
11. Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER 1999 Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci USA* 96:7986–7991
12. Wilson JD, Lasnitzki I 1971 Dihydrotestosterone formation in fetal tissues of the rabbit and rat. *Endocrinology* 89:659–668
13. Siiteri PK, Wilson JD 1974 Testosterone formation and metabolism during male sexual differentiation in the human embryo. *J Clin Endocrinol Metab* 38:113–125
14. Imperato-McGinley J, Zhu YS 2002 Androgens and male physiology the syndrome of 5 α -reductase-2 deficiency. *Mol Cell Endocrinol* 198:51–59
15. Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B, Vaughan ED 1992 Comparison of the effects of the 5 α -reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. *Endocrinology* 131:1149–1156
16. Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM 1988 Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* 240:327–330
17. Zhou ZX, Lane MV, Kempainen JA, French FS, Wilson EM 1995 Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. *Mol Endocrinol* 9:208–218
18. McPhaul MJ 2002 Androgen receptor mutations and androgen insensitivity. *Mol Cell Endocrinol* 198:61–67
19. Quigley CA 2002 The postnatal gonadotropin and sex steroid surge: insights from the androgen insensitivity syndrome. *J Clin Endocrinol Metab* 87:24–28
20. Tsuji M, Shima H, Cunha GR 1991 *In vitro* androgen-induced growth and morphogenesis of the Wolffian duct within urogenital ridge. *Endocrinology* 128:1805–1811
21. Brinkmann AO 2001 Molecular basis of androgen insensitivity. *Mol Cell Endocrinol* 179:105–109
22. Majdic G, Millar MR, Saunders PT 1995 Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. *J Endocrinol* 147:285–293
23. Bentvelsen FM, Brinkmann AO, van der Schoot P, van der Linden JE, van der Kwast TH, Boersma WJ, Schroder FH, Nijman JM 1995 Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Mol Cell Endocrinol* 113:245–253

24. Cunha GR, Alarid ET, Turner T, Donjacour AA, Boutin EL, Foster BA 1992 Normal and abnormal development of the male urogenital tract. Role of androgens, mesenchymal-epithelial interactions, and growth factors. *J Androl* 13:465–475
25. Arnold JT, Kaufman DG, Seppala M, Lessey BA 2001 Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. *Hum Reprod* 16:836–845
26. Donjacour AA, Cunha GR 1991 Stromal regulation of epithelial function. *Cancer Treat Res* 53:335–364
27. Buchanan DL, Kurita T, Taylor JA, Lubahn DB, Cunha GR, Cooke PS 1998 Role of stromal and epithelial estrogen receptors in vaginal epithelial proliferation, stratification, and cornification. *Endocrinology* 139:4345–4352
28. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y 1987 The endocrinology and developmental biology of the prostate. *Endocr Rev* 8:338–362
29. Fisher JS, Macpherson S, Marchetti N, Sharpe RM 2003 Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. *Hum Reprod* 18:1383–1394
30. Bowman CJ, Turner KJ, Sar M, Barlow NJ, Gaido KW, Foster PM 2005 Altered gene expression during rat Wolffian duct development following di(n-butyl) phthalate exposure. *Toxicol Sci* 86:161–174
31. Foster PM, Harris MW 2005 Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. *Toxicol Sci* 85:1024–1032
32. McIntyre BS, Barlow NJ, Foster PM 2001 Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci* 62:236–249
33. Mylchreest E, Sar M, Cattley RC, Foster PM 1999 Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156:81–95
34. Jost A, Vigier B, Prepin J, Perchellet JP 1973 Studies on sex differentiation in mammals. *Recent Prog Horm Res* 29:1–41
35. Stinnakre MG 1975 [Period of sensitivity to androgens of the Wolff duct of the rat fetus]. *Arch Anat Microsc Morphol Exp* 64:45–59 (French)
36. Barlow NJ, Foster PM 2003 Pathogenesis of male reproductive tract lesions from gestation through adulthood following in utero exposure to di(n-butyl) phthalate. *Toxicol Pathol* 31:397–410
37. Cooke PS, Young P, Cunha GR 1991 Androgen receptor expression in developing male reproductive organs. *Endocrinology* 128:2867–2873
38. Jirsova Z, Vernerova Z 1993 Involution of the Wolffian duct in the rat. *Funct Dev Morphol* 3:205–206
39. Dyche WJ 1979 A comparative study of the differentiation and involution of the Mullerian duct and Wolffian duct in the male and female fetal mouse. *J Morphol* 162:175–209
40. Habert R, Rouiller-Fabre V, Lecerf L, Levacher C, Saez JM 1992 Developmental changes in testosterone production by the rat testis in vitro during late fetal life. *Arch Androl* 29:191–197
41. Drews U, Dieterich HJ 1978 Cell death in the mosaic epididymis of sex reversed mice, heterozygous for testicular feminization. *Anat Embryol (Berl)* 152:193–203
42. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev* 16:271–321
43. Turner KJ, McIntyre BS, Phillips SL, Barlow NJ, Bowman CJ, Foster PM 2003 Altered gene expression during rat Wolffian duct development in response to in utero exposure to the antiandrogen linuron. *Toxicol Sci* 74:114–128
44. Cunha GR, Young P 1992 Role of stroma in oestrogen-induced epithelial proliferation. *Epithelial Cell Biol* 1:18–31
45. Cunha GR, Hayward SW, Dahiya R, Foster BA 1996 Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. *Acta Anat (Basel)* 155:63–72
46. Aupperle H, Schoon D, Schoon HA 2004 Physiological and pathological expression of intermediate filaments in the equine endometrium. *Res Vet Sci* 76:249–255
47. Schlatt S, Weinbauer GF, Arslan M, Nieschlag E 1993 Appearance of α -smooth muscle actin in peritubular cells of monkey testes is induced by androgens, modulated by follicle-stimulating hormone, and maintained after hormonal withdrawal. *J Androl* 14:340–350
48. Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB, Cunha GR 1997 Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *Proc Natl Acad Sci USA* 94:6535–6540
49. Cunha GR, Bigsby RM, Cooke PS, Sugimura Y 1985 Stromal-epithelial interactions in adult organs. *Cell Differ* 17:137–148
50. Marker PC, Donjacour AA, Dahiya R, Cunha GR 2003 Hormonal, cellular, and molecular control of prostatic development. *Dev Biol* 253:165–174

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The Critical Time Window for Androgen-Dependent Development of the Wolffian Duct in the Rat

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Androgens are thought to separately regulate stabilization and differentiation of the Wolffian duct (WD), but the time windows for these effects are unclear. To address this, fetal rats were exposed to flutamide within either an early window (EW) [embryonic day 15.5 (E15.5) to E17.5], when the WD degenerates in the female, or a later window (LW) (E19.5–E21.5), when the WD morphologically differentiates in the male, or during the full window of WD development (FW) (E15.5–E21.5). WDs were examined for abnormalities during fetal (E21.5) or postnatal life, and anogenital distance and prostate presence/absence were recorded. Exposure to FW- or EW-flutamide, but not to LW-flutamide, induced comparable abnormalities in the fetal WD at E21.5, namely reduced WD coiling, reduced cell proliferation, reduced epithelial cell height, altered epithelial vimentin expression, and reduced expression of smooth mus-

cle actin in the WD inner stroma. Exposure to EW- or FW-flutamide, but not to LW-flutamide, resulted in incomplete/absent WDs in more than 50% of males by adulthood, although such abnormalities were infrequent in fetal life. These findings suggest that androgen action during the EW is sufficient to promote WD morphological differentiation several days later. Because the androgen receptor is expressed in the WD stroma but not in the epithelium during this EW, WD differentiation is likely to be dependent on androgen-mediated signaling from the stroma to the epithelium. In conclusion, the critical window for androgen action in regulating WD development in the rat is between E15.5 and E17.5. This window is also important for prostate formation and anogenital distance masculinization. (*Endocrinology* 148: 0000–0000, 2007)

DURING MAMMALIAN DEVELOPMENT, before activation of SRY within the somatic cells of the genital ridge, the urogenital tract is initially identical in both sexes, comprising the Müllerian duct (MD) and the Wolffian duct (WD) (1, 2). In males, after differentiation of the different somatic cell types in the fetal testis, the Sertoli cells secrete anti-Müllerian hormone to induce MD degeneration, whereas the Leydig cells secrete testosterone, which acts via the androgen receptor (AR) to stabilize and rescue the WD (2, 3). Conversely, in females, the ovaries do not synthesize either androgens or anti-Müllerian hormone so the WD degenerates and the MD persists (1–3). In males, once the WD is stabilized, it then differentiates to form its adult derivatives; the cranial portion of the WD convolutes to form the adult epididymis, the central portion remains a relatively simple straight duct and forms the vas deferens, whereas the seminal vesicles bud off the distal segment (4, 5). This process is thought to be controlled by androgens because XY males with inactivating mutations in the AR have a female phenotype with intraabdominal testes, no prostate, and a lack of WD-derived tissues (6–9). In the rat, epididymal differentiation becomes evident between embryonic day 19.5 (E19.5) to E21.5 and is characterized by transformation of the cranial

segment of the simple straight WD into an intensely coiled structure (10). It is presumed that the patterning of this transformation is under the control of testosterone (11, 12), the secretion of which begins at approximately E15.5 in the fetal rat and peaks at E19.5 (13).

Previous researchers have shown that interfering with androgen action during fetal life, using either AR mutant animals or exposure to antiandrogenic compounds, results in abnormal reproductive tracts in adult males (for review, see Refs. 14–21). Most studies have exposed rats to antiandrogenic compounds between E13 and E21, encompassing the onset of testosterone production by the fetal testis and the window of male reproductive development, and have then examined the effects of this in adulthood. In such males, the epididymis and/or vas deferens is often incomplete or absent altogether. It has been presumed that this was attributable to a failure of the male WD to stabilize early in fetal development, as occurs naturally in females. However, we demonstrated recently that exposure of pregnant rats to 100 mg/kg flutamide during the period E15.5–E21.5 did not induce regression of the male WD but did result in subsequent impairment of WD morphological differentiation (10). These studies and those reported previously in the literature suggest that fetal WD development may be a biphasic process, with stabilization and differentiation possibly under differential control. It has been presumed that androgen action is vital for both early stabilization of the WD and the later segmentation and differentiation of the WD into its adult derivatives, but the precise window of androgen action in WD development remains unknown.

One previous study in the mouse showed that exposure to hydroxyflutamide from E11 to E15 resulted in smaller epi-

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Abbreviations: AGD, Anogenital distance; AR, androgen receptor; E, embryonic day; EW, early window; FW, full window; LW, late window; MD, Müllerian duct; NGS, normal goat serum; PBST, PBS with 0.1% Tween 20; PND, postnatal day; SMA, smooth muscle actin; WD, Wolffian duct.

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didymides and infertility, whereas exposure to hydroxyflutamide from E19 to E20 did not result in any obvious reproductive abnormalities or infertility in adulthood (22). A similar study using rats examined the effects of maternal exposure to a single dose of flutamide (50 mg/kg) on E16, E17, E18, or E19 and showed that this treatment resulted in a similar range of reproductive abnormalities in adulthood as did exposure to flutamide throughout the period of reproductive development (E12–E21) but at a lower incidence (23). Exposure of pregnant rats to another antiandrogen, vinclozolin (400 mg/kg), between E14 and E19 resulted in malformations of the external genitalia, permanent nipples, reduced anogenital distance (AGD), and reduced seminal vesicle, ventral prostate, and epididymal weights. When vinclozolin treatment was restricted to 2-d periods (e.g. E14–E15 or E18–E19), similar abnormalities were observed, but the incidence and severity was reduced and no obvious abnormalities were reported in adult WD-derived structures (24).

There is thus no consensus as to when androgens act to 1) stabilize the WD and 2) induce its differentiation (i.e. epididymal coiling) and establish the patterning of the segmentation of the WD into its adult derivatives. It is also unclear as to when a lack of fetal androgen action may result in loss of part or all of the WD and precisely when this loss first manifests because the studies to date have only examined adults. To address this deficit in understanding, we used a model system in which fetal rats were exposed to flutamide specifically within either an early window (EW), when the WD degenerates in the female (E15.5–E17.5), or in a later window (LW), when the WD morphologically differentiates in the male (E19.5–E21.5); the impact on WD development in fetal (E21.5) and postnatal [postnatal day 17 (PND17), PND42, and PND70] life was then studied. We previously published evidence highlighting WD development as a biphasic event with WD stabilization and differentiation dependent on either different levels of androgen action or on different mechanisms (10). The present study sought to establish the critical window of androgen action in both these processes and has found, surprisingly, that androgen action within the same early time window (E15.5–E17.5) is sufficient to induce both rescue and later morphological differentiation of the WD.

Materials and Methods

Animals

Wistar rats were bred and maintained in our own animal house under standard conditions according to United Kingdom Home Office guidelines. Animals had access *ad libitum* to water and a soy-free breeding diet (SDS, Dundee, UK). Time matings were established, and the presence of a vaginal plug was taken as evidence of mating; this was defined as E0.5.

Treatment and collection of tissues and measurement of AGD

A total of 36 pregnant dams were used for this study, with dams randomly allocated to treatment groups. Dams were dosed daily by oral gavage between 0830 and 1000 h with flutamide (Sigma, Poole, UK) at 100 mg/kg in 1 ml/kg corn oil/2.5% DMSO (Sigma); these doses were selected based on previously reported results (10, 17). Dosing was undertaken during critical windows that either 1) encompassed the period of WD stabilization, defined by the timing of WD regression in females (our unpublished data) (E15.5–E17.5, EW-flutamide, *n* = 7 litters) or 2)

encompassed the period of WD morphological differentiation, defined by the appearance of coiling in the future epididymal segment of the WD (E19.5–E21.5, LW-flutamide, *n* = 8 litters) (Fig. 1). Flutamide exposure between E17.5 and E19.5 was not examined in this study because we aimed to examine the role for androgens during WD stabilization or during WD morphological differentiation rather than to investigate the role for androgens during the entire period of reproductive development.

To compare the effects of short window flutamide exposure with the previously published effects of exposure to flutamide from the onset of androgen production by the testis until birth [E15.5–E21.5, full window (FW)-flutamide, *n* = 6 litters] (10), WDs from FW-flutamide exposed males were included in all analyses. To reduce animal usage, these WDs were taken from both archived datasets (10) as well as from litters generated specifically for this study; there were no obvious differences between the archived (*n* = 8 litters) and newly generated datasets (*n* = 23 litters). Control dams (*n* = 10 litters) were gavaged daily with the vehicle alone at 1 ml/kg corn oil/2.5% DMSO. Dams were checked daily for signs of toxicity, and dam weights were recorded daily throughout the dosing regimen. Male offspring were subsequently evaluated in late fetal life (E21.5) or at PND17, PND42, or PND70.

For recovery of fetal animals, dams were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Pups were recovered, decapitated, and placed in ice-cold PBS (Sigma). Postnatal animals (PND17, PND42, and PND70) were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Before recovery of reproductive tracts, AGD was measured in fetal (E21.5) and postnatal animals using digital calipers (Faithfull Tools, Kent, UK), because it is widely believed that AGD reflects the degree of masculinization of the animal (for review, see Ref. 25). Female littermates were also examined for AGD.

Our previous studies have shown that WD morphological differentiation is well established by E21.5, as evidenced by coiling in the future epididymal segment of the WD (10). WDs were collected from male pups by microdissection, examined with a Leica (Nussloch, Germany) MZ6 dissecting microscope and photographed using a Leica ICA camera to enable gross morphological evaluation and measurement of WD luminal length (see below). This study focused on the development of the future epididymis and vas deferens (i.e. the upper portions of the WD) rather than the seminal vesicles, which are believed to depend on both testosterone and dihydrotestosterone. For each pup, the future epididymis and vas deferens segments of one isolated WD was snap frozen in liquid nitrogen for subsequent RNA and protein analysis by Taqman and Western blot, respectively, whereas the other was fixed in Bouin's fixative for 1 h before being transferred into 70% ethanol and processed for 17.5 h in an automated Leica TP1050 processor for later use in histological analysis. Fixed WDs were embedded horizontally in paraffin wax, sectioned (5 μ m), floated onto slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma), and dried overnight at 50 C before histological analysis (see below). Representative WDs from at least three animals from at least three litters from the aforementioned treatment groups were subsequently used for the studies detailed below.

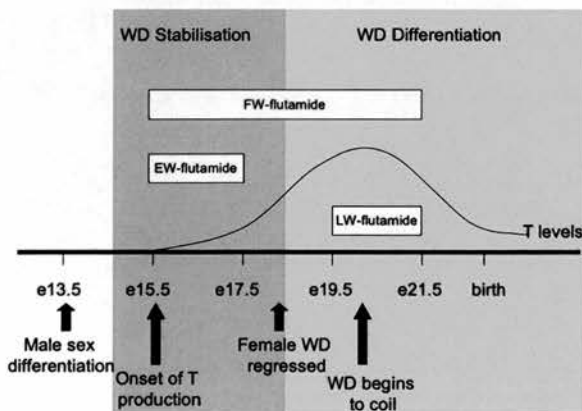


FIG. 1. Summary of duration of flutamide exposure in relation to key events in reproductive development and testosterone (T) levels in the fetal male testis (4, 13, 38, 39).

To recover postnatal reproductive tracts, the abdomen of the supine male rat was opened, and testes, epididymides, and vas deferens were pulled out of the scrotal sac by the fat pad and removed from the animal.

Gross morphology and histological analysis

Reproductive tracts from control and flutamide-exposed males at E21.5 or PND17, PND42, and PND70 were analyzed microscopically, at the time of dissection, for any gross morphological abnormalities. Histological analysis was performed on WD sections stained with hematoxylin and eosin, using standard protocols. Note was taken of any histological abnormalities, including swollen lumens and epithelial malformation.

WD luminal length measurement

Differentiation of WDs at E21.5 was quantified by measuring the luminal length of WD from control and treated animals on photographs taken of WD at the time of dissection, as detailed previously (10). A line was digitally drawn through the lumen of the WD image taken at time of dissection using the NIH Image J program. To ensure reproducibility, luminal length was measured for WDs from 15–30 individuals from at least three different litters per treatment group.

Immunohistochemistry

Immunohistochemistry was performed on Bouin's-fixed WDs recovered from control and treated fetuses at E21.5 using previously published standard avidin-peroxidase protocols (10). The antibodies used for immunohistochemistry, their dilutions, and sources are listed in Table 1. Cellular sites of expression of AR, smooth muscle actin (SMA), and pan-cytokeratin were determined, and slides were photographed using a Provis AX70 (Olympus Optical, London, UK) microscope fitted with a Canon DS6031 camera (Canon Europe, Amsterdam, The Netherlands). To ensure reproducibility of results and allow accurate comparison of immunostaining between treatment groups, sections of WDs from control and treated animals were processed in parallel on at least three occasions; sections of WDs from at least three animals in each treatment group were run on each occasion. Appropriate negative controls were included, whereby the primary antibody was replaced by normal goat serum (NGS) alone, to ensure that any staining observed was specific; none of the antibodies used showed other than minor nonspecific staining.

Double-fluorescence immunohistochemistry for cytokeratin and vimentin

Sections from Bouin's-fixed WDs were deparaffinized and rehydrated, and antigen retrieval was performed as detailed previously (10). Nonspecific binding sites were blocked by incubating sections in NGS (Autogen Bioclear UK Ltd., Wiltshire, UK) diluted 1:4 in PBS containing 5% BSA (Sigma) for 1 h and then incubated overnight at 4°C with anti-pan-cytokeratin antibody (Sigma) diluted 1:200 in NGS/PBS/BSA. This signal was detected by incubating sections for 1 h with goat anti-mouse secondary antibody directly conjugated with Alexa Fluor 546 (Invitrogen, Carlsbad, CA) diluted 1:200 in PBS to produce red fluorescence. Sections were washed well in PBS before blocking in NGS/PBS/BSA for 1 h. Because the anti-vimentin antibody (DakoCytomation, Ely, UK) and the pan-cytokeratin antibody were both raised in mice, the anti-vimentin antibody was directly labeled with Alexa Fluor 488 using an Alexa Fluor 488 labeling monoclonal antibody labeling kit (Invitrogen). This antibody was diluted 1:15 in NGS/PBS/BSA and incubated on sections for 2 h at room temperature. Because no secondary antibody

detection was required, sections were counterstained for 10 min with a nuclear-specific blue fluorescent label (4',6'-diamidino-2-phenylindole; Sigma) diluted 1:1000 in PBS and then mounted in Mowiol mounting medium (Calbiochem, Lutterworth, UK). Fluorescent images were captured using a Zeiss (Welwyn Garden City, UK) LSM 510 Meta Axiovert 100M confocal microscope.

Frequency of cell mitoses in WDs

One randomly selected section from WDs recovered from control and flutamide-exposed fetuses at E21.5 was immunostained for phospho-histone H3 (Upstate Biotechnology, Dundee, UK) using a Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK) as published previously (10). Phospho-histone H3-positive cells were counted in the epithelial compartment and the inner and outer stromal layers of the WD using an Olympus Optical (Tokyo, Japan) BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments, Cambridge, UK). Image-Pro Plus version 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics, Wokingham, Berkshire, UK) was used as detailed previously (10). Positive cells were counted only in the future epididymal portion of the WD, and the number of positive cells was then related to the amount of epithelium seen in section and the total length of the WD lumen (10). Sections from 4–14 fetuses from three to five separate litters were analyzed from both control and flutamide-exposed animals.

Epithelial cell height measurement

WDs (E21.5) from six control fetuses and four to six fetuses from each of the flutamide treatment groups were sectioned and immunostained for pan-cytokeratin as detailed above to clearly label all epithelial cells. The software and stereological equipment noted above were used. Using a $\times 63$ objective, epithelial cell height was measured in every fifth epithelial cell per section. This was performed separately for the caput, corpus, and caudal regions of the future epididymal portion of the WD (10). Only epithelial cells in which the nucleus could be clearly identified were measured, thus excluding from analysis any epithelial cells from the flutamide treatment groups that were severely flattened or disintegrating.

Western blot analysis

Protein was harvested from frozen WDs using radioimmunoprecipitation assay lysis buffer as published previously (10). Protein concentration was determined using a Bio-Rad (Hemel Hempstead, UK) bicinchoninic acid protein assay kit according to the instructions of the manufacturer, and 20 μ g of each protein extract was loaded onto a 7.5% polyacrylamide gel. Gels were subjected to electrophoresis at 100 V under reducing conditions. The proteins were then electrotransferred overnight at 20 V onto nitrocellulose membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were washed in PBS before incubating in Odyssey blocking buffer (LI-COR, Lincoln, NE) diluted 1:1 in PBS for 1 h at room temperature to block nonspecific binding sites. Membranes were incubated overnight at 4°C with anti-SMA antibody (Sigma) diluted 1:10,000 and anti- β -tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:300 in Odyssey blocking buffer/PBS. The LI-COR buffer allows detection of more than one antibody at a time; therefore, both antibodies could be added at once as long as their host species differed, so they could be discriminated by secondary antibodies of different specificities. The anti- β -tubulin antibody was used as a standardization loading control. Residual primary antibody was washed off with PBS with 0.1% Tween 20 (PBST) (Sigma) for 5 min (three times). Membranes were then incubated for 1 h at room temperature with the

TABLE 1. Antibodies: source, dilution, retrieval, and species

Antibody	Source	Dilution	Retrieval	Species raised in
AR	Santa Cruz Biotechnology	1:50	Citrate	Rabbit
SMA	Sigma	1:10000	None	Mouse
Pan-cytokeratin	Sigma	1:200	Citrate	Mouse
Phospho-histone H3	Upstate Biotechnology	1:1000	Citrate	Rabbit
Vimentin	DakoCytomation	1:1000	Citrate	Mouse

secondary antibodies diluted 1:5000 in Odyssey blocking buffer/PBST. Goat antirabbit secondary antibody conjugated with IRDye 800 (Rockland, Gilbertsville, PA) was used to detect β -tubulin, whereas goat antimouse secondary antibody conjugated with Alexa Fluor 680 (Invitrogen) was added to detect SMA. Membranes were again washed in PBST for 5 min (three times) and then PBS (one time) to remove any residual Tween 20, before detecting the signal. The membrane was scanned using the LI-COR buffer, according to the instructions of the manufacturers.

Antibody specificity was confirmed by the detection of only one band at the expected size when visualizing each antibody. The intensity of the bands was then quantified, with the area of exposure equating to the amount of labeled protein present in the sample. Protein expression level was corrected for loading using β -tubulin. To ensure reproducibility of results, the Western blot was performed at least twice, and protein was isolated from at least three WDs from two to three different litters each time.

Statistical analysis

Values have been expressed as means \pm SEM. Data were analyzed using one-way ANOVA, followed by the Bonferroni's *post hoc* test, using GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

Results

Effects of flutamide exposure on AGD and the reproductive tract

Maternal exposure to flutamide during the entire window of reproductive development (E15.5–E21.5, FW-flutamide) prevented normal masculinization of fetal external genitalia, with AGD significantly reduced ($P < 0.001$) at E21.5 and PND17 to a length comparable with that of control females. Exposure to flutamide (100 mg/kg) early in WD development (E15.5–E17.5, EW-flutamide) reduced AGD by the same amount as did FW-flutamide exposure, whereas maternal exposure to flutamide late in WD development (E19.5–

E21.5, LW-flutamide) did not result in any significant change in male AGD at E21.5 or PND17 when compared with age-matched control males (Fig. 2).

Maternal exposure to FW-flutamide (E15.5–E21.5) or EW-flutamide (E15.5–E17.5) prevented normal prostate and penis formation in males and resulted in the presence of a vaginal pouch, whereas exposure to LW-flutamide (E19.5–E21.5) had no such effects (data not shown).

Effects on fetal and postnatal WD development of impaired androgen action during critical windows of development

Fetal. Exposure to FW-flutamide reduced WD development and coiling compared with age-matched controls, as published previously (10). Exposure to EW-flutamide inhibited coiling (WD differentiation) at E21.5 to a similar extent as did FW-flutamide exposure, whereas exposure to LW-flutamide had no obvious effect on WD coiling at E21.5 compared with age-matched controls (Fig. 3). This difference was confirmed quantitatively (Fig. 3), highlighting that there was no significant difference in luminal length between WDs from litters exposed to FW-flutamide and those exposed to EW-flutamide. Exposure to LW-flutamide did not significantly reduce WD luminal length compared with age-matched controls.

As well as the reduction in coiling, exposure to FW-flutamide resulted in some WDs appearing incomplete at E21.5 (Table 2), with thinning of the epithelium and missing corpus/cauda segments, as reported previously (10). This phenotype of missing segments was not noted at E21.5 in fetuses exposed to either EW- or LW-flutamide (Table 2).

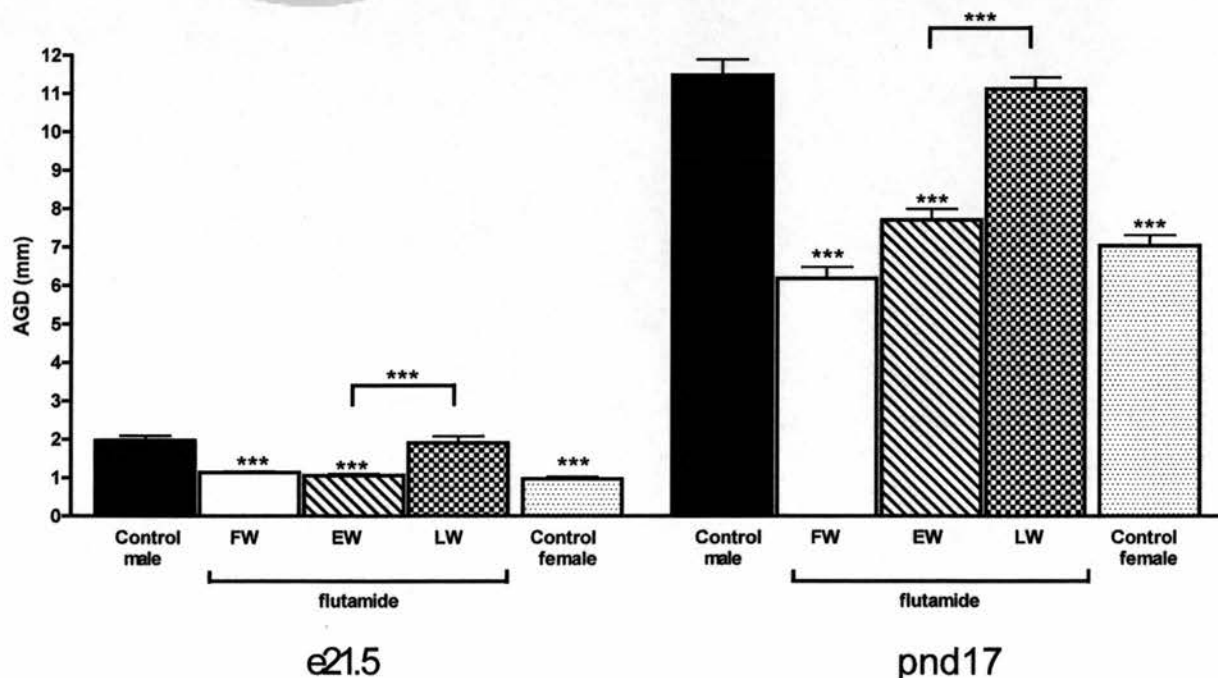
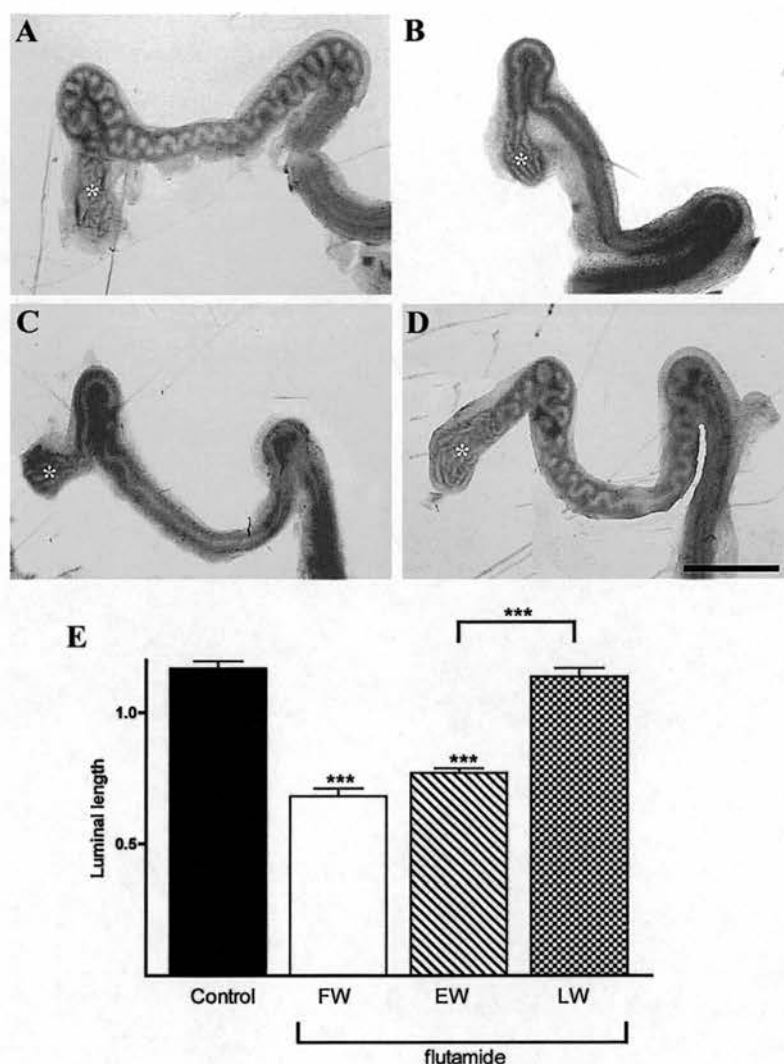


FIG. 2. AGD in males and females from control and flutamide-exposed litters at E21.5 and PND17. Note that AGD was significantly smaller in control females than in males; AGD in males exposed to flutamide between E15.5 and E21.5 (FW; white bars) or between E15.5 and E17.5 (EW; striped bars) was reduced compared with control males and was comparable with female AGD. Exposure to LW-flutamide (E19.5–E21.5; checkered bars) did not affect male AGD. ***, $P < 0.001$ compared with control male AGD. Values are means \pm SEM for 6–23 animals.

FIG. 3. Representative WDs at E21.5 from control and flutamide-exposed fetuses. Note that coiling is evident in the future epididymal segment of the control WD (A) and that exposure to flutamide between E15.5 and E21.5 (FW; B) reduced this coiling. Exposure to flutamide between E15.5 and E17.5 (EW; C) reduced WD coiling to a similar extent as did exposure to FW-flutamide (B). In contrast, exposure to LW-flutamide (E19.5–E21.5; D) did not obviously reduce WD coiling compared with age-matched controls. * indicates efferent ducts. Scale bar, 1 mm. E shows quantification of coiling (luminal length) of E21.5 WDs from control and flutamide-exposed litters. Note the significant reduction in WD luminal length in animals exposed *in utero* to FW-flutamide (white bar) or EW-flutamide (striped bar) when compared with controls (black bar). Exposure to LW-flutamide (checkered bar) did not reduce WD luminal length. Values are mean \pm SEM for 15–30 animals per group. **, $P < 0.01$ compared with exposure to flutamide from E15.5 to E17.5; ***, $P < 0.001$ compared with the respective control value.



Postnatal. Consistent with our findings in the fetus and with previous reports (23, 26, 27), FW- or EW-flutamide exposure resulted in abnormal morphology of the adult male reproductive tract with underdeveloped or incomplete epididymides, lack of a vas deferens, and underdeveloped or vestigial seminal vesicles. At PND17 (early puberty), all males exposed to either EW- or LW-flutamide treatment had complete epididymides and vasa deferentia, but, by late puberty (PND42), 40% of males exposed to EW-flutamide had incomplete reproductive tracts with missing or incomplete epididymides and/or vasa deferentia (Table 2). By adulthood (PND70), 50% of males exposed to EW-flutamide had incomplete epididymides and/or vasa deferentia (Table 2); an additional 25% of such males had un-

derdeveloped or abnormal epididymides/vasa deferentia (data not shown). This phenotype was similar to that observed in males exposed to FW-flutamide, in which 83% of males examined in adulthood had incomplete WD-derived tissues (Table 2). In contrast, males exposed to LW-flutamide all had complete epididymides and vasa deferentia at PND42 and in adulthood (Table 2).

AR expression

Androgens act through the AR; therefore, it was important to establish whether flutamide exposure affected AR expression. In control animals, AR expression was initially re-

TABLE 2. Percentage of rats with an intact WD (E21.5) or epididymis and vas deferens (*i.e.* WD-derived tissues, PND17, PND42, adult) after exposure to flutamide (100 mg/kg) during specific time windows in fetal life

Age	Treatment group			
	Control (%)	FW-flutamide (%)	EW-flutamide (%)	LW-flutamide (%)
E21.5	100 (34 of 34)	89 (34 of 38)	100 (30 of 30)	100 (19 of 19)
PND17 (early puberty)	100 (32 of 32)	87 (7 of 8)	100 (3 of 3)	100 (5 of 5)
PND42 (late puberty)	100 (12 of 12)	37 (3 of 8)	60 (4 of 6)	100 (4 of 4)
Adult	100 (18 of 18)	17 (2 of 12)	50 (4 of 8)	100 (3 of 3)

Values are for at least three litters per treatment/age, and the number of animals is shown in parentheses.

stricted to the WD stroma at E15.5 (Fig. 4) and E16.5 (data not shown), but, by E17.5, a few epithelial cells were weakly immunopositive for AR (Fig. 4). AR was expressed in many epithelial cells by E19.5 and in the majority of epithelial cells by E21.5 (Fig. 4). Exposure to either EW- or LW-flutamide did not alter AR expression in either the stroma or the epithelium at E21.5 or PND17 (data not shown). Additional analysis of AR expression using Taqman quantitative RT-PCR confirmed that there was no significant difference in AR mRNA expression in flutamide-exposed WDs at E21.5 compared with controls (data not shown). This is in agreement with results reported previously, showing that FW-flutamide exposure did not alter AR expression in the fetal WD (10) or postnatally (our unpublished data).

Frequency of cell mitoses in WDs

Cell proliferation was detected in all compartments of WDs from both control and flutamide-exposed animals at E21.5. Immunostaining for phospho-histone H3 detected fewer proliferating cells in WDs from pups exposed to FW- or EW-flutamide compared with age-matched controls, but there was no apparent change in WDs exposed to LW-flutamide (data not shown). This was confirmed quantitatively (Fig. 5). Cell proliferation data were expressed as the overall number of proliferating cells in the complete epididymal portion of the WD rather than per 100 μ m epithelium, so as to take into account any treatment-induced changes in WD length.

Reduction in epithelial cell height after flutamide exposure

Exposure to FW-flutamide resulted in epithelial abnormalities in some areas of the WD in some animals at E21.5,

for example, shorter epithelium, missing cells, and widened lumens, as reported previously (10). These abnormalities were also evident in WDs from animals exposed to EW-flutamide, but histological examination revealed no obvious abnormalities in the epithelium of WDs from fetuses exposed to LW-flutamide compared with age-matched controls (Fig. 6). These differences in WD epithelial cell height at E21.5 were confirmed quantitatively (Fig. 6).

Vimentin expression

Vimentin protein expression was examined in control and flutamide-exposed WDs because vimentin is an early mesenchymal intermediate filament, the expression of which is lost as cells undergo differentiation; aberrant expression of vimentin can result in abnormal cell behavior (28, 29). Vimentin was immunolocalized to the WD stroma of both control and flutamide-exposed fetuses at all ages examined (Fig. 7). Vimentin was also detected at the basolateral edges of epithelial cells in the WD at E17.5; this “spiking” was seen along the full length of the WD at E15.5 (data not shown) and E17.5 (Fig. 7A). In contrast, at E19.5 and E21.5, vimentin expression was rarely seen in the epithelium of the caput region in control WDs (Fig. 7, B and C), although it was still evident in the epithelium of the caudal region at both E19.5 and E21.5 (data not shown). Exposure to flutamide did not affect expression of vimentin in the stroma but affected its distribution in the epithelium (Fig. 7). For example, vimentin spiking was apparent in the epithelium at the caput region as well as in the caudal region (Fig. 7) in WDs from E21.5 fetuses exposed to FW- or EW-flutamide; this spiking was rarely seen at the caput region of WDs from fetuses exposed to LW-flutamide (Fig. 7) but was still noted in the epithelium

FIG. 4. Immunoexpression of AR in the developing WD from control animals at E15.5–E21.5. AR (immuno-stained brown) was confined to the WD stroma at E15.5 (A, B). AR was first detected in a few epithelial cells at E17.5 (C, D) and was strongly expressed in the epithelium by E19.5 (E, F). AR was intensely expressed in both the stroma and the epithelium by E21.5 (G, H). Scale bars, 50 μ m.

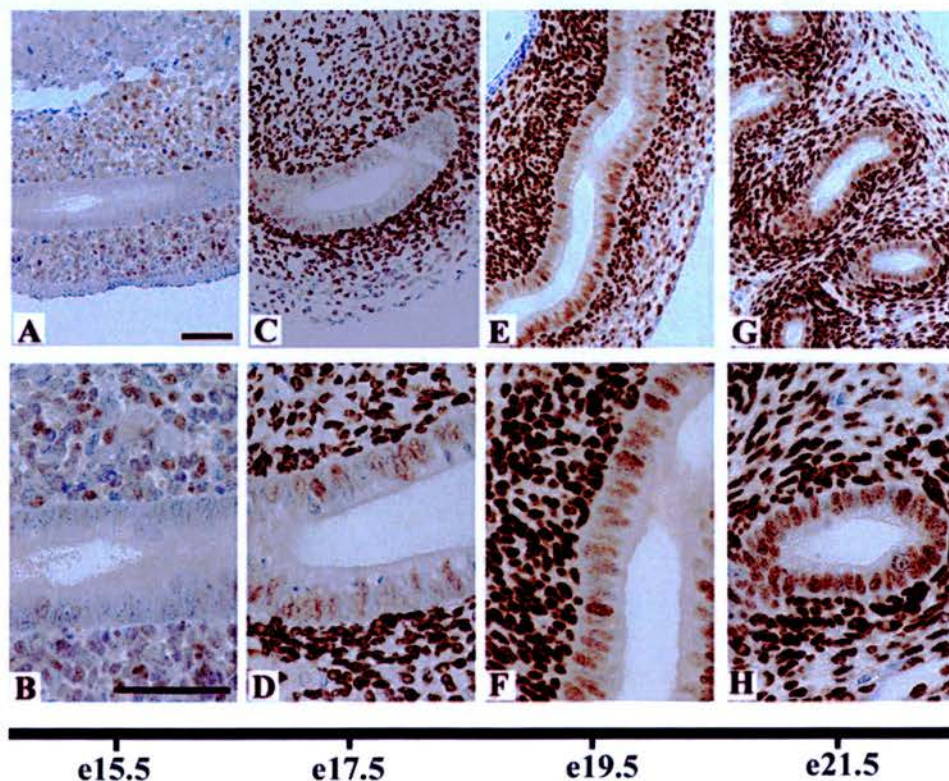
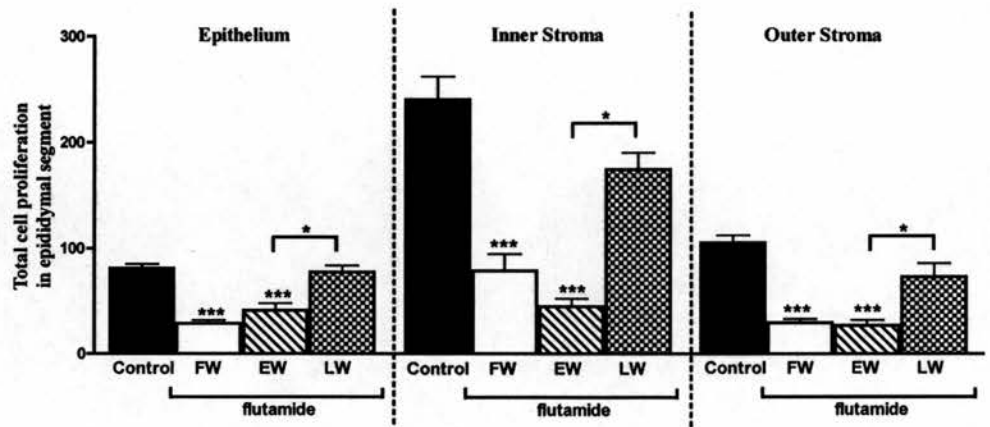


FIG. 5. Total number of mitotic epithelial and stromal cells in the entire epididymal segment of the WD at E21.5 from control and flutamide-exposed fetuses. Note the significant reduction in the number of mitotic cells in WDs from animals exposed to either FW-flutamide (white bars) or EW-flutamide (striped bars) compared with controls (black bars); this was apparent in all cellular compartments. Note also that exposure to LW-flutamide (checkered bars) did not affect WD cell proliferation at E21.5 compared with age-matched controls; these values were significantly higher than those from EW-flutamide WDs. *, $P < 0.05$ compared with respective EW-flutamide value; ***, $P < 0.001$ compared with respective control value. Values are means \pm SEM for 3–16 animals per group.



of the caudal region, as in age-matched controls (data not shown).

Evidence for flutamide effects on inner stromal cells

Immunostaining for SMA, which is expressed in the inner stromal compartment and believed to be androgen dependent (10, 30), revealed a reduction in the SMA-positive layer in WDs from E21.5 fetuses that had been exposed to FW- or EW-flutamide compared with age-matched controls (Fig. 8). No obvious change was noted in this SMA-positive layer in WDs from males exposed to LW-flutamide (Fig. 8D). The SMA-positive stromal compartment varied considerably in breadth between sections of WDs, so the reduction in SMA protein expression in WDs recovered from fetuses exposed to FW- or EW-flutamide was confirmed quantitatively by Western blot analysis (Fig. 8E). This confirmed that SMA protein levels in WDs from EW- and FW- treatments, but not those from LW-flutamide fetuses, were significantly different from that in age-matched controls (Fig. 8E).

Discussion

It is widely accepted that androgens play a critical role in WD development. A recent study published by our group confirmed the role for androgens in male fetal reproductive development and highlighted WD development as a biphasic process, with fetal exposure to flutamide (100 mg/kg) inhibiting development, elongation, and differentiation of the WD but not its earlier stabilization (10). Abnormalities in WD-derived structures persisted and worsened by adulthood, confirming that the patterning of the reproductive tract is established in the fetus and that interfering with androgen action during this critical period permanently impairs reproductive tissues. It has been assumed that androgen-driven gene activity is essential during the period of both WD stabilization and morphological differentiation, but the precise time window within which androgens act in males to mediate WD stabilization and/or differentiation remains unclear. In the present study, we used an *in utero* model system in which fetal rats were exposed to flutamide on either E15.5–E17.5 (EW), to interfere with androgen action during the “stabilization window” when the WD is degenerating in the

female, or E19.5–E21.5 (LW), during the “differentiation window” when WD morphologically differentiates into the epididymis in the male. The impact of these treatments on WD morphology and functional differentiation in fetal (E21.5) and postnatal (PND17, PND42, and PND70) life was also investigated.

The main findings of these studies are, first, that androgen action during the early time window (E15.5–E17.5), which is several days before any sign of WD morphological differentiation, is sufficient to promote later WD segmentation and development (coiling/functional differentiation). Second, exposure to EW-flutamide was able to induce similar gross and histological abnormalities in the fetal and adult WD as those observed after exposure to FW-flutamide. Because the AR is expressed in the WD stroma but not in the epithelium during this “early” window, this suggests that AR-dependent androgen action must signal via the stroma to pattern later WD development and differentiation. Finally, exposure to flutamide during the “late” window (E19.5–E21.5), the period in which WD morphological differentiation actually occurs, did not result in any obvious gross or histological WD abnormalities at this or later times, thus suggesting that the pattern of WD segmentation is already established by E19.5 and that its morphological differentiation is no longer dependent on high levels of androgen action. The critical window for high levels of androgen action in establishing the pattern of WD development in rats is therefore between E15.5 and E17.5.

Because exposure to FW-flutamide did not prevent stabilization of the male WD but impaired WD differentiation (*i.e.* coiling) between E19.5 and E21.5 (10), we anticipated that reducing androgen action during WD morphological differentiation would allow WD “rescue” but would prevent subsequent WD differentiation, whereas exposure to EW-flutamide would not have any major impact on WD coiling or functional differentiation. Contrary to these expectations, reduced androgen action early in male reproductive tract development (E15.5–E17.5) resulted in a similar phenotype to that documented after exposure to FW-flutamide, with males having a vaginal pouch instead of a normal prostate, impaired WD development, and an AGD the same as that in

normal females. This phenotype was not observed in male fetuses exposed to LW-flutamide. These results suggest that prostate formation, WD patterning, and masculinization of AGD is established between E15.5 and E17.5, several days before any sign of morphological differentiation of any of these tissues, and that high levels of androgen action later in fetal development are not essential for maintaining this male phenotype. These findings support and extend those of a recent study in which pregnant rats were exposed to a single dose of flutamide (50 mg/kg) between E16 and E19, and the impact on male phenotype of adult rats was examined. The results revealed that exposure to flutamide on E16 or E17 resulted in missing epididymides in adults, whereas exposure on E18 or E19 only resulted in smaller epididymides (23). These authors highlighted that the peak incidence of abnormal prostate development was noted in adults exposed to flutamide on E17 or E18, similar to our observations in males exposed to flutamide between E15.5 and E17.5 (23). Furthermore, they noted that exposure to a single dose of flutamide on any one day between E16 and E19 resulted in a similar range of reproductive abnormalities as did exposure to flutamide throughout the period of reproductive development (E12–E21), although the incidence was lower (23). We also noted a reduced incidence of reproductive abnormalities after exposure to EW-flutamide compared with that observed in animals exposed to FW-flutamide in animals examined at a range of different ages.

It was surprising that high levels of androgen action did not appear to be essential during the window of morphological differentiation of the WD (E19.5–E21.5). Instead, it appears that, once the pattern of WD segmentation has been

established by earlier androgen exposure, the WD continues to develop normally, even if androgen action is reduced during the period of morphological differentiation. It is worth noting that, as reported previously (10), exposure to this level of flutamide may not completely block androgen action; therefore, we cannot completely rule out a role for low levels of androgens during this LW of WD development (E19.5–E21.5). Flutamide was used to interfere with androgen action because it has been shown to be a strong competitive AR antagonist and exposure during fetal development interrupts male reproductive development (17, 26, 27, 31, 32). Flutamide is believed to be cleared from the body relatively quickly and has a plasma half-life of 5–6 h in man (33), suggesting that fetuses are not exposed to flutamide for more than 24 h after the final gavage. Studies in the mouse have shown that exposure to flutamide on E19 and E20 did not result in any obvious reproductive abnormalities or infertility in adulthood, whereas exposure on E11–E15 or E15–E20 resulted in smaller epididymides and infertility or lack of a prostate, respectively (22). These studies again confirm the long-term deleterious effects of EW short-term androgen blockade. It remains unclear what role, if any, androgens play late in fetal male reproductive development, and additional investigation of more endpoints would be required in males from litters exposed to flutamide between E19.5 and E21.5.

In the present study and as reported previously (10), exposure to flutamide during the FW of male reproductive development (E15.5–E21.5) resulted in incomplete WDs in 11% of males at E21.5, 50% of males at PND17, and 83% of adult males. This is in agreement with previous studies

FIG. 6. Representative gross abnormalities seen in the epithelium of WDs (immunostained brown for cytokeratin) from control (A) and flutamide-exposed (B–D) fetuses at E21.5. Note the apparent reduction in epithelial cell height (arrow) in WDs from males exposed to FW-flutamide (B) or EW-flutamide (C) compared with age-matched controls (A). This was not obvious in WDs from males exposed to LW-flutamide (D). Scale bars, 100 μ m. E shows quantification of epithelial cell height in WDs at E21.5 from males exposed to FW-flutamide (white bar) or EW-flutamide (striped bar) or LW-flutamide (checkered bar) compared with age-matched controls (black bar). Values are means \pm SEM for 3–19 WDs per age/treatment, each from different litters. *, $P < 0.05$ and ***, $P < 0.001$ compared with E21.5 controls.

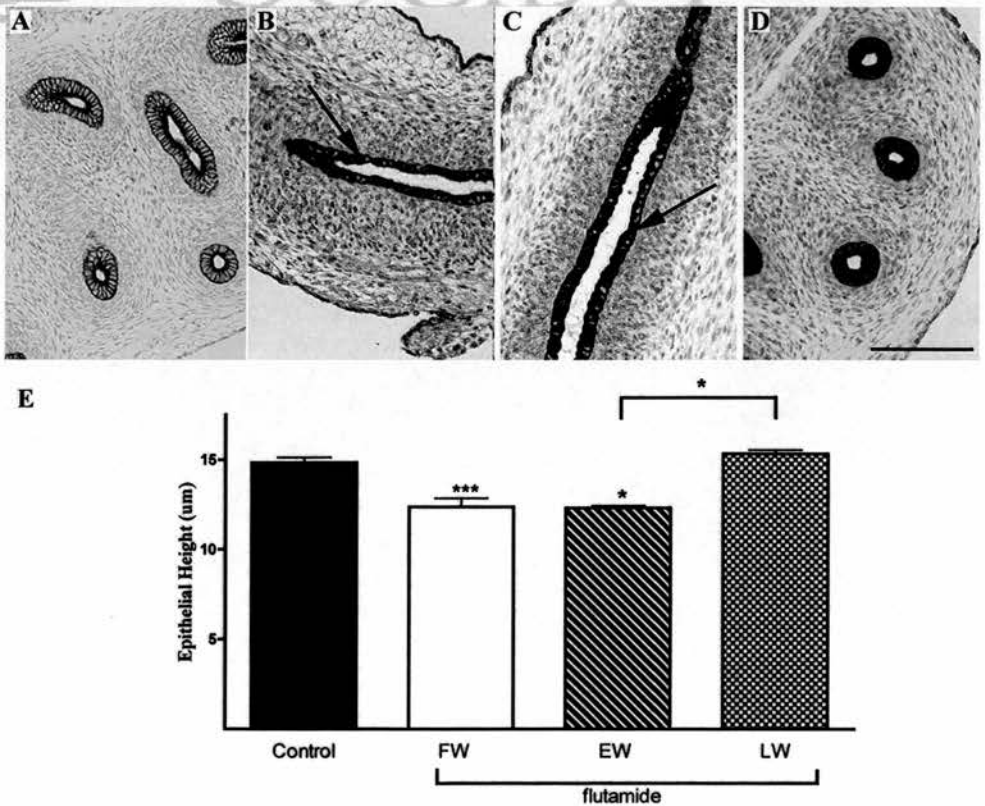
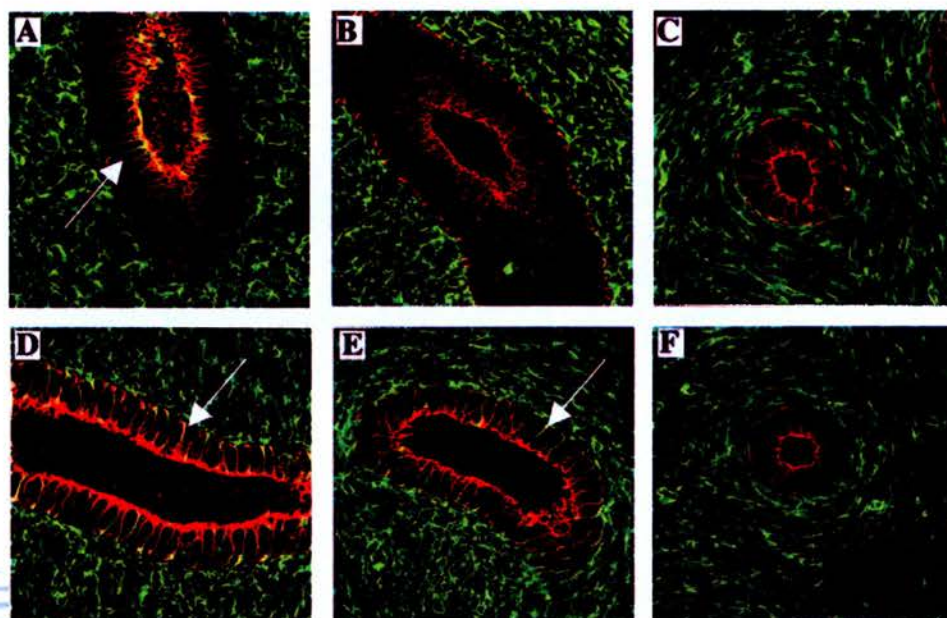


FIG. 7. Coexpression of vimentin (green) and cytokeratin (red) protein in the caput region of WDs from control and flutamide-exposed animals. Note that vimentin was expressed in the stroma at E17.5 (A), E19.5 (B), and E21.5 (C–F) and did not vary with flutamide treatment (D, FW-flutamide; E, EW-flutamide; F, LW-flutamide). Vimentin was also detected at the basolateral edges of the WD epithelium (arrow) at E17.5 (A) but was absent by E19.5 (B) and E21.5 (C) in control animals. However, vimentin spiking in the epithelium was still apparent in the caput region of the WD at E21.5 in fetuses exposed to FW-flutamide (D) or EW-flutamide (E) but was not evident in WDs exposed to LW-flutamide (F).



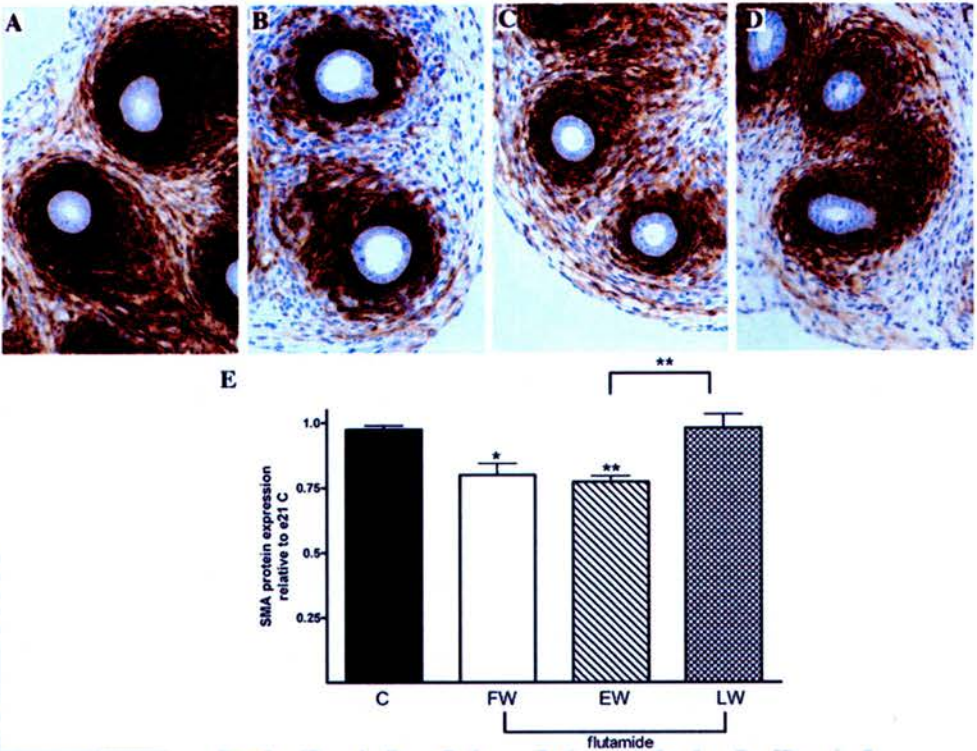
showing that exposure to antiandrogenic compounds *in utero* results in a high frequency of epididymal malformations when studied in adulthood (17, 23, 26, 27, 34). Exposure to LW-flutamide did not result in incomplete WDs at any time point studied. In contrast, EW-flutamide did not show evidence of loss of WD during fetal life, but 40% of pubertal males (PND42) and 50% of adults had incomplete epididymides and/or vasa deferentia. This has provided compelling evidence that androgen action early in WD development (E15.5–E17.5) is essential to establish the pattern of WD development and that interfering with this has long-term consequences for the adult male reproductive tract. However, it remains to be shown why completion of puberty should result in delayed degeneration or loss of WD-derived tissues in animals exposed to EW- or FW-flutamide.

To confirm that the disturbances in WD development seen in animals exposed to flutamide during the early treatment window involved similar mechanisms to those seen in animals exposed to flutamide throughout reproductive development (10), various endpoints were examined (summarized in Table 3). As we reported previously (10), exposure to flutamide between E15.5 and E21.5 did not alter expression of the AR in the stroma or epithelium of the WD at any age during fetal life or postnatally (our unpublished data). Similarly, in the present study, exposure to flutamide during either defined window (early or late) did not interrupt AR expression in the fetus or at PND17, confirming that these WDs are still capable of responding to androgens; because AR is expressed in the postnatal (PND17, PND42, and PND70) WD derivatives from animals exposed to flutamide between E15.5 and E21.5 (our unpublished data), it is unlikely that exposure to flutamide during the EW or LW would alter AR expression. This is in contrast to findings by Bentvelsen *et al.* (31), who were unable to detect AR protein by immunohistochemistry in WDs from E21.5 fetuses exposed to 100 mg/kg flutamide. This difference may be attributable to differences in rat strain or to the use of different anti-AR antibodies and immunohistochemistry techniques.

Exposure to FW-flutamide resulted in reduced epithelial cell height and abnormal epithelial development in the WD by E21.5 (10). The same epithelial disturbance was not found in WDs from males exposed to LW-flutamide but was seen in WDs from males exposed to EW-flutamide. These effects on the epithelium were not evident until E21.5, suggesting that, although the epithelium initially forms normally, flutamide exposure results in subsequent abnormalities during differentiation, possibly attributable to impaired androgen-driven signaling between the stroma and epithelium. As well as the impact of flutamide exposure on epithelial differentiation, we reported previously that exposure to flutamide between E15.5 and E21.5 impaired WD stromal cell differentiation, as evidenced by a reduction in SMA protein expression at E19.5–E21.5 (10). In the present studies, a similar reduction was noted in WDs from fetuses exposed to flutamide during the EW but not the LW of WD development, highlighting the a role for androgens between E15.5 and E17.5 in regulating stromal cell differentiation as well as epithelial cell differentiation.

Vimentin, an early mesenchymal intermediate filament, was expressed in the WD stroma at all ages studied (E15.5–E21.5), and its expression in the stroma was not obviously affected by flutamide exposure. This stromal vimentin is likely to be providing mechanical support to the developing WD and may also mediate signaling through interactions with and organization of the cytoskeleton (35). Vimentin was also expressed on the basolateral boundary of the epithelial cells in some regions of the developing WD. It is thought that vimentin expression often precedes expression of other intermediate filaments during differentiation, for example desmin. Vimentin is initially expressed in the epithelium along the full length of the WD at E15.5 but becomes progressively more restricted such that, by E21.5, it is confined to the caudal region in control WDs. This may mean that vimentin expression in the epithelium is replaced by another intermediate filament in a craniocaudal direction as development proceeds. At E21.5, epithelial spiking of vimentin

FIG. 8. Comparative expression of SMA at E21.5 in WDs from controls (A) and animals exposed to FW-flutamide (B), EW-flutamide (C), or LW-flutamide (D). Note the apparent reduction in SMA immunorexpression (brown) in WDs from fetuses exposed to FW- or EW-flutamide but not in WDs from fetuses exposed to LW-flutamide. E shows quantitative data from Western blot analysis comparing expression of SMA protein at E21.5 in WDs from control (C) and flutamide-exposed animals. Note the reduced levels of SMA protein in WDs from fetuses exposed to FW-flutamide (white bar) or EW-flutamide (striped bar), although no obvious difference can be seen in SMA protein expression in WDs from fetuses exposed to LW-flutamide (checkered bar) compared with age-matched controls (black bar). n = 3–5 WDs per age/treatment, from at least three different litters. Loading was corrected using β -tubulin protein expression.



was detected along the full length of the epididymal segment of WDs from fetuses exposed to FW- or EW-flutamide rather than just in the cauda. This pattern is in contrast to WDs from fetuses exposed to LW-flutamide, which were similar to controls. The alteration in epithelial vimentin expression could be attributable to failure of the epithelial cells to differentiate and thus to switch off epithelial vimentin expression and may mean that these epithelial cells retain some mesenchymal-like properties. This might explain the reduced epithelial cell height and loss of attachment to the basement membrane seen in flutamide-exposed WDs. Vimentin is required for cell motility, and increased vimentin expression is seen in prostate cancer cells as they become more invasive (28). It could therefore be hypothesized that these vimentin-positive WD epithelial cells may be gaining motility but there is no definitive evidence for this, and additional investigation of earlier and later time points might provide some insight. Studies in the equine endometrium have shown that, in cases of endometriosis, cytokeratin and vimentin become coexpressed in the epithelium, and it has been suggested that this could be attributable to increased proliferation and a loss of

cell to cell contact (36). Other researchers have also shown that altered hormone action could induce epithelial vimentin expression (37).

It is concluded that the pattern of WD coiling and its subsequent ability to develop fully during postnatal life is established by androgen action early in fetal reproductive development (E15.5–E17.5) and that, by E19.5, androgen-dependent WD patterning is already established and is no longer dependent on high levels of androgen action. Reduced androgen action during this early time window inhibited coiling at E21.5 to the same extent as did exposure from E15.5 to E21.5 and resulted in a similar high incidence of epididymal loss/abnormalities in late puberty and adulthood. Exposure later in fetal life (E19.5–E21.5), the period in which WD morphological differentiation actually occurs, did not impact on WD coiling at E21.5 or on subsequent epididymal development postnatally. The critical window for high levels of androgen action in establishing the pattern of WD development is therefore between E15.5 and E17.5 in the rat.

TABLE 3. Summary of endpoints affected by exposure to flutamide during specific time windows in fetal life compared with controls at E21.5

Endpoint	Treatment		
	FW-flutamide	EW-flutamide	LW-flutamide
AGD	↓	↓	↔
WD coiling	↓	↓	↔
AR expression	↔	↔	↔
Cell proliferation	↓	↓	↔
Epithelial cell height	↓	↓	↔
SMA expression	↓	↓	↔
Vimentin caput epithelial expression	↑	↑	↔

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M.W., P.T.K.S., and R.M.S. have nothing to declare.

References

1. Drews U 2000 Local mechanisms in sex specific morphogenesis. *Cytogenet Cell Genet* 91:72–80
2. George FW, Wilson J 1994 The physiology of reproduction. 2nd ed. New York: Raven Press
3. Huhtaniemi I 1994 Fetal testis—a very special endocrine organ. *Eur J Endocrinol* 130:25–31
4. Wilson JD, George FW, Griffin JE 1981 The hormonal control of sexual development. *Science* 211:1278–1284
5. Robaire B, Hermon L 1988 Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: *Physiology of reproduction*. 2nd ed. New York: Raven Press
6. Quigley CA 2002 Editorial: the postnatal gonadotropin and sex steroid surge—insights from the androgen insensitivity syndrome. *J Clin Endocrinol Metab* 87:24–28
7. Tsuji M, Shima H, Cunha GR 1991 *In vitro* androgen-induced growth and morphogenesis of the Wolffian duct within urogenital ridge. *Endocrinology* 128:1805–1811
8. Brinkmann AO 2001 Molecular basis of androgen insensitivity. *Mol Cell Endocrinol* 179:105–109
9. McPhaul MJ 2002 Androgen receptor mutations and androgen insensitivity. *Mol Cell Endocrinol* 198:61–67
10. Welsh M, Saunders PT, Marchetti NJ, Sharpe RM 2006 Androgen-dependent mechanisms of Wolffian duct development and their perturbation by flutamide. *Endocrinology* 147:4820–4830
11. Veysiere G, Berger M, Jean-Faucher C, de Turkheim M, Jean C 1982 Testosterone and dihydrotestosterone in sexual ducts and genital tubercle of rabbit fetuses during sexual organogenesis: effects of fetal decapitation. *J Steroid Biochem* 17:149–154
12. George FW, Wilson J 1994 Gonads and ducts in mammals. In: Knobil E, Neill JD, eds. *The physiology of reproduction*. 2nd ed. New York: Raven Press
13. Warren DW, Halmeyer GC, Eik-Nes KB 1972 Synthesis and metabolism of testosterone in the fetal rat testis. *Biol Reprod* 7:94–99
14. Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E, Guillello L 2001 Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update* 7:248–264
15. Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X, Xing L, Boyce BF, Hung MC, Zhang S, Gan L, Chang C 2002 Generation and characterization of androgen receptor knockout (ARKO) mice: an *in vivo* model for the study of androgen functions in selective tissues. *Proc Natl Acad Sci USA* 99:13498–13503
16. Lyon MF, Hawkes SG 1970 X-linked gene for testicular feminization in the mouse. *Nature* 227:1217–1219
17. Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B, Vaughan ED 1992 Comparison of the effects of the 5 α -reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. *Endocrinology* 131:1149–1156

18. Mylchreest E, Cattley RC, Foster PM 1998 Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci* 43:47–60
19. McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PM 2000 Effects of *in utero* exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat. *Toxicol Appl Pharmacol* 167:87–99
20. Foster PM, Mylchreest E, Gaido KW, Sar M 2001 Effects of phthalate esters on the developing reproductive tract of male rats. *Hum Reprod Update* 7:231–235
21. McIntyre BS, Barlow NJ, Sar M, Wallace DG, Foster PM 2002 Effects of *in utero* linuron exposure on rat Wolffian duct development. *Reprod Toxicol* 16:131–139
22. Silversides DW, Price CA, Cooke GM 1995 Effects of short-term exposure to hydroxyflutamide *in utero* on the development of the reproductive tract in male mice. *Can J Physiol Pharmacol* 73:1582–1588
23. Foster PM, Harris MW 2005 Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. *Toxicol Sci* 85:1024–1032
24. Wolf CJ, LeBlanc GA, Ostby JS, Gray Jr LE 2000 Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. *Toxicol Sci* 55:152–161
25. Edwards TM, Moore BC, Guillello L 2006 Reproductive dysgenesis in wildlife: a comparative view. *Int J Androl* 29:109–121
26. McIntyre BS, Barlow NJ, Foster PM 2001 Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci* 62:236–249
27. Mylchreest E, Sar M, Cattley RC, Foster PM 1999 Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156:81–95
28. Singh S, Sadacharan S, Su S, Beldegrun A, Persad S, Singh G 2003 Overexpression of vimentin: role in the invasive phenotype in an androgen-independent model of prostate cancer. *Cancer Res* 63:2306–2311
29. Paranko J, Virtanen I 1986 Epithelial and mesenchymal cell differentiation in the fetal rat genital ducts: changes in the expression of cytokeratin and vimentin type of intermediate filaments and desmosomal plaque proteins. *Dev Biol* 117:135–145
30. Schlatt S, Weinbauer GF, Arslan M, Nieschlag E 1993 Appearance of α -smooth muscle actin in peritubular cells of monkey testes is induced by androgens, modulated by follicle-stimulating hormone, and maintained after hormonal withdrawal. *J Androl* 14:340–350
31. Bentvelsen FM, Brinkmann AO, van der Schoot P, van der Linden JE, van der Kwast TH, Boersma WJ, Schroder FH, Nijman JM 1995 Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Mol Cell Endocrinol* 113:245–253
32. Hotchkiss AK, Ostby JS, Vandenberg JG, Gray Jr LE 2002 Androgens and environmental antiandrogens affect reproductive development and play behavior in the Sprague-Dawley rat. *Environ Health Perspect* 110 (Suppl 3):435–439
33. Neri RO, Monahan M 1972 Effects of a novel nonsteroidal antiandrogen on canine prostatic hyperplasia. *Invest Urol* 10:123–130
34. Turner KJ, McIntyre BS, Phillips SL, Barlow NJ, Bowman CJ, Foster PM 2003 Altered gene expression during rat Wolffian duct development in response to *in utero* exposure to the antiandrogen linuron. *Toxicol Sci* 74:114–128
35. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD 1994 Molecular biology of the cell. New York: Garland Publishing
36. Aupperle H, Schoon D, Schoon HA 2004 Physiological and pathological expression of intermediate filaments in the equine endometrium. *Res Vet Sci* 76:249–255
37. Klymkowsky MW, Bachant JB, Domingo A 1989 Functions of intermediate filaments. *Cell Motil Cytoskeleton* 14:309–331
38. Habert R, Picon R 1984 Testosterone, dihydrotestosterone and estradiol-17 β levels in maternal and fetal plasma and in fetal testes in the rat. *J Steroid Biochem* 21:193–198
39. Tapanainen J, Kellokumpu-Lehtinen P, Pelliniemi L, Huhtaniemi I 1981 Age-related changes in endogenous steroids of human fetal testis during early and midpregnancy. *J Clin Endocrinol Metab* 52:98–102